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CONTENTS

Cytopathic Changes in Tissue Cultures Derived from a Pig Infected with Hog Cholera 165
D. P. Gustafson and C. M. Pomerat

Some Pathological Effects of Anterior Pituitary Growth Hormone Preparations on Swine 174
L. P. Doyle, E. J. Turman, and F. N. Andrews

Exudative Epidermitis of Pigs 179
L. D. Jones

Airborne Transmissible Gastroenteritis 194
E. F. Reber

Fescue Lameness in Cattle. I. Experimental Production of the Disease 196
R. Jensen, A. W. Deem, and D. Knaus

Fescue Lameness in Cattle. II. Ergot Alkaloids in Tall Fescue Grass 202
D. D. Maag and J. W. Tobiska

Reports of Liver Glycogen Determinations by Biopsies on Dairy Cows 205
C. W. Raker and J. A. Rogers

Effects of Dietary Trace Minerals, Excess Calcium, and Various Roughages on Weight Gains of Hereford Heifers 207
M. J. Swenson, G. K. L. Underbjerg, and D. D. Goetsch

The Utilization of Intravenously Administered Glucose, Invert Sugar, and Fructose in Cattle 213
D. D. Goetsch, G. K. L. Underbjerg, and M. J. Swenson

The Course and Pathogenicity of Initial Infections with *Dictyocaulus Viviparus*, the Lungworm of Cattle 217
R. Rubin and J. T. Lucker

The Resistance of Milk Samples to the Activity of *Streptococcus Agalactiae* as Affected by Pasture 227
W. D. Pounden, N. A. Frank, and W. C. Krauss

The Resistance of Milk Samples to the Activity of *Streptococcus Agalactiae* as Affected by Feeding Silage 231
W. D. Pounden, N. A. Frank, R. W. Brown, and R. K. Scherer

An Agent Enhancing Listeriosis of Sheep 235
C. Olson and D. Segre

Phenothiazine Treatment of Fattening Lambs in Drylot 243
P. H. Allen and J. E. Oldfield

(Continued on page 163)

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Volume XVII APRIL, 1956 No. 63

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(Contents continued from cover)

An Observation on the Relationship of Cobalt Deficiency to Internal Parasites of Sheep 246
W. L. Threlkeld, N. O. Price, and W. N. Linkous

Effects of Trace Minerals, Dicalcium Phosphate, Phenothiazine, and Combinations of These on the Resistance of Lambs to *Haemonchus Contortus* and Other Nematodes 252
R. F. Shumard, R. J. Emerick, W. E. Bemrick, C. A. Herrick, A. L. Pope, and P. H. Phillips

Immunizing Activity of Alum-Precipitated Protective Antigen of *Bacillus Anthracis* in Cattle, Sheep, and Swine..... 256
A. S. Schlingman, H. B. Devlin, G. G. Wright, R. J. Maine, and M. C. Manning

Serological Differentiation of the Equine Abortion Virus from the Human and Swine Influenza, Mumps, and Newcastle Disease Viruses..... 262
E. R. Doll, W. H. McCollum, J. T. Bryans, E. W. Crowe

Agglutination of Horse Erythrocytes by Tissue Extracts from Hamsters Infected with Equine Abortion Virus..... 267
W. H. McCollum, E. R. Doll, and J. T. Bryans

Studies on the Depression of Radioiodine Uptake by the Thyroid After Phenothiazine Administration. II. Effect of Phenothiazine on the Horse Thyroid..... 271
B. F. Trum and R. H. Wasserman

The Static Intravascular Extracapillary Blood Volume of the Kidney of the Dog as Estimated by the Vinylite Corrosion Technique..... 276
L. M. Julian

Continuous Phenothiazine Therapy of Dogs Artificially Infected with *Ancylostoma Caninum* 279
R. R. Bell

The Transmission of Visceral Lymphomatosis with Live Virus Newcastle Disease Vaccines 283
B. R. Burmester, C. H. Cunningham, G. E. Cottral, R. C. Belding, R. F. Gentry

Preparation of a New Pleuropneumonia-like Organism Antigen for the Diagnosis of Chronic Respiratory Disease by the Agglutination Test..... 290
H. E. Adler and R. Yamamoto

Interference Between Newcastle Disease and Infectious Bronchitis Viruses..... 294
L. E. Hanson, F. H. White, and J. O. Alberts

Attempts to Influence Listeriosis in Chickens Using Antimetabolites..... 299
D. Segre, C. Olson, C. L. Marsh, and I. C. Blore

Studies on Errington's Disease in Muskrats. I. Pathological Changes..... 303
G. H. Lord, A. C. Todd, and C. Kabat

Studies on Errington's Disease in Muskrats. II. Etiology..... 307
G. H. Lord, A. C. Todd, C. Kabat, and H. Mathiak

Studies of Prepared Feeds in Relation to Salmonella Infection in Laboratory Animals. II. Reproduction and Growth of Mice on Heated and Unheated Commercially Prepared Feeds 311
C. A. Griffin and W. R. Thompson

The Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine..... 318
J. R. Douglas, N. F. Baker, and W. M. Longhurst

The Influence of Environmental Conditions on the Growth and Dissociation of *Brucella Abortus* 324
E. Sanders and I. F. Huddleson

Physiological Characteristics Differentiating *Vibrio Fetus* and Other Vibrios..... 331
C. D. Kuzdas and E. V. Morse

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VOL. XVII APRIL, 1956 No. 63

Cytopathic Changes in Tissue Cultures Derived from a Pig Infected with Hog Cholera

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THERE has been renewed interest in cytopathogenic effects of viruses in tissue culture following the report by Enders *et al.*[2] that degenerative changes accompanied the propagation of poliomyelitis virus in extraneural body tissues. Recent reviews emphasize the attention accorded this subject.

Lynn and Morgan[4] interpreted cytopathogenicity to be visible cellular changes, gross or microscopic, such as distortion and pyknosis of the nucleus, increased granularity of cytoplasm, irregularity of cell shape, and cellular disintegration, which occur as a result of viral proliferation; however, their review does not include agents producing only inclusion bodies and no other visible change. The viruses are grouped as those having marked, moderate, or no cytopathogenic activity.

In the review by Enders,[1] cytopathogenicity is considered to be ". . . the capacity to induce any demonstrable departure from the normal either in the morphological or functional properties of cells," and three types of activity are suggested although two or more types might appear successively within the same cell population. Changes due to the presence of virus are designated as (*a*) those causing only degeneration, manifested by rounding or assumption of irregular or bizarre outlines, granulation of cytoplasm, swelling of the cell, nuclear swelling, pyknosis, fragmentation of nuclear chromatin, cell death, and disintegration; (*b*) those causing formation of inclusion bodies and cell degeneration; and (*c*) those causing formation of multi-nucleated cells or syncytial masses and degeneration with or without inclusion bodies and exhibiting a tendency to produce "foci" of infected cells.

Reports of hog cholera virus in tissue cultures, other than those in which cell survival time is prolonged in a Maitland-type culture, are quite limited. In none of the available literature was there any comment on cytopathogenic effects of this virus on the cultured cells.

This work deals with observations of the apparent effects of hog cholera virus on tissue cultures from the natural host which had become sick following inoculation with the virus and whose tissues were the source of the virus.

MATERIALS AND METHODS

Swine.—Three litter mate pigs, 8 weeks old, were obtained from a farm near College Station, Texas. It was estimated that swine had been raised on the farm for 20 years without any having been vaccinated against hog cholera; consequently, these were likely to be susceptible.

The pigs were placed 1 in each of three wire cages with separate feeding and watering facilities. Two of the 3 were inoculated intramuscularly with a virulent strain of hog cholera virus.* Morning temperatures were recorded daily. On the sixth postinoculation day, 1 of the inoculated pigs and the uninoculated one were destroyed. Tissues were removed for culture and a routine macroscopic examination was made of each pig.

Tissue Cultures.—Primary cultures were made of the following tissues from both pigs: cerebellum, wall of the third ventricle, corpus callosum, liver, thymus, heart, pituitary, lung, bone marrow, and spleen. In addition, cultures were made from the choroid plexus of the infected pig. The explanted tissues were cut approximately 1.0 mm.

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* Virus obtained from the Indiana Farm Bureau Cooperative Association, Inc., Thorntown, Ind.

square. Three of these explants were mounted in a chicken plasma and chicken embryo extract clot on each 12 by 50-mm. glass cover slip and two cover slips were placed back to back in a 16 by 150-mm. pyrex culture tube and stoppered with cotton. These were then allowed to remain undisturbed at room temperature for about three hours. Ten tubes were prepared for each tissue, the six explants per tube making 60 of each tissue available for observation. Two milliliters of a nutrient medium consisting of 50 per cent human ascitic fluid, 45 per cent Gey's solution containing 0.2 mg. per cent phenol red, 5 per cent chicken embryo extract, and 1,000 units of penicillin per milliliter were added to each tube. The tubes were then placed in a roller drum revolving at approximately eight rotations per hour in an incubator at approximately 37 C. Hydrogen ion concentrations were approximated by color comparisons of tubes containing tissue cultures with La-Motte phenol red standards. At various intervals, cultures of each tissue were fixed and stained in the technique suggested by Jacobson.[5]

Phase microscopy was utilized to permit both still photographic and time-lapse cinematographic recordings of salient conditions.

RESULTS

The temperatures recorded (table 1) indicate the susceptibility of the pigs to hog cholera virus and the course of the disease

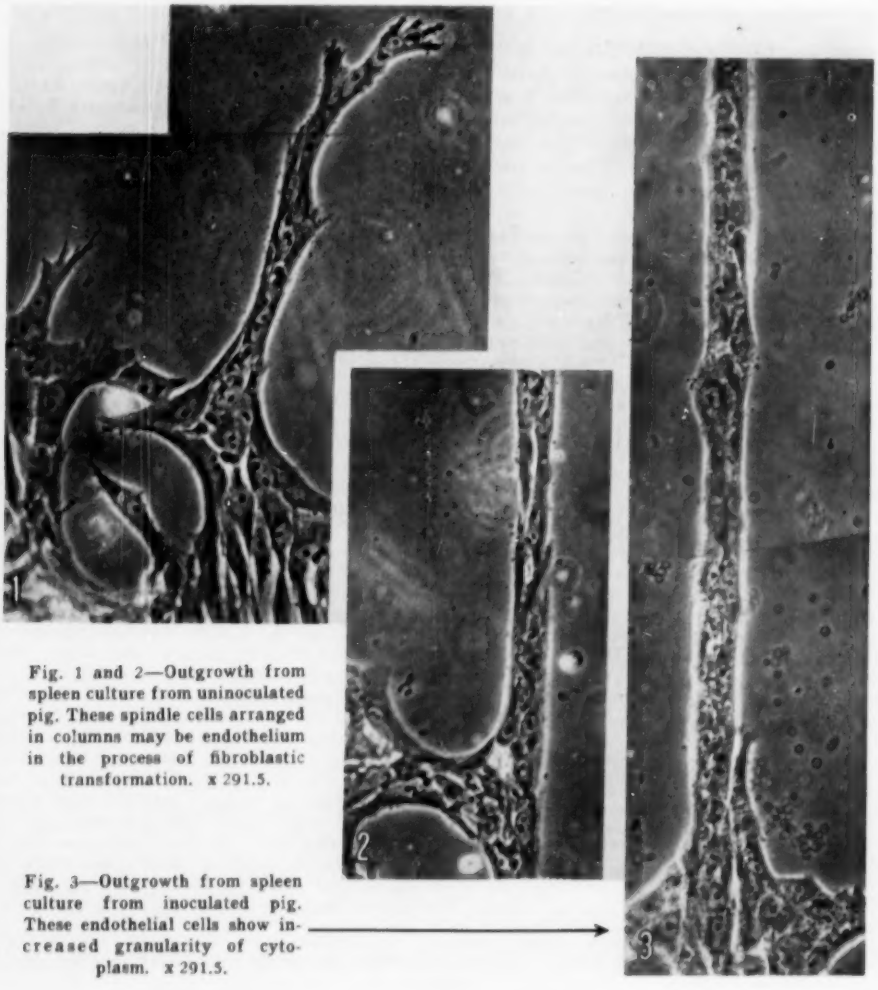


Fig. 1 and 2—Outgrowth from spleen culture from uninoculated pig. These spindle cells arranged in columns may be endothelium in the process of fibroblastic transformation. x 291.5.

Fig. 3—Outgrowth from spleen culture from inoculated pig. These endothelial cells show increased granularity of cytoplasm. x 291.5.

until six days postinoculation. Necropsy of the 2 pigs revealed further evidence of the presence of disease in the inoculated pig as compared to the uninoculated pig. The inoculated pig showed diffuse hemorrhages of the spleen, punctate subserous hemorrhages of the kidneys, similar hemorrhages in the bladder, peripheral hemorrhages of the mandibular, mediastinal, mesenteric, prefemoral, and popliteal lymph nodes, and infarcts of the lungs. Also there was inflammation of the gastrointestinal tract. The uninoculated pig was apparently normal.

Differences in pH (table 2) were slight and not of use in distinguishing cultures from the inoculated pig from those of the uninoculated one. Records were not made

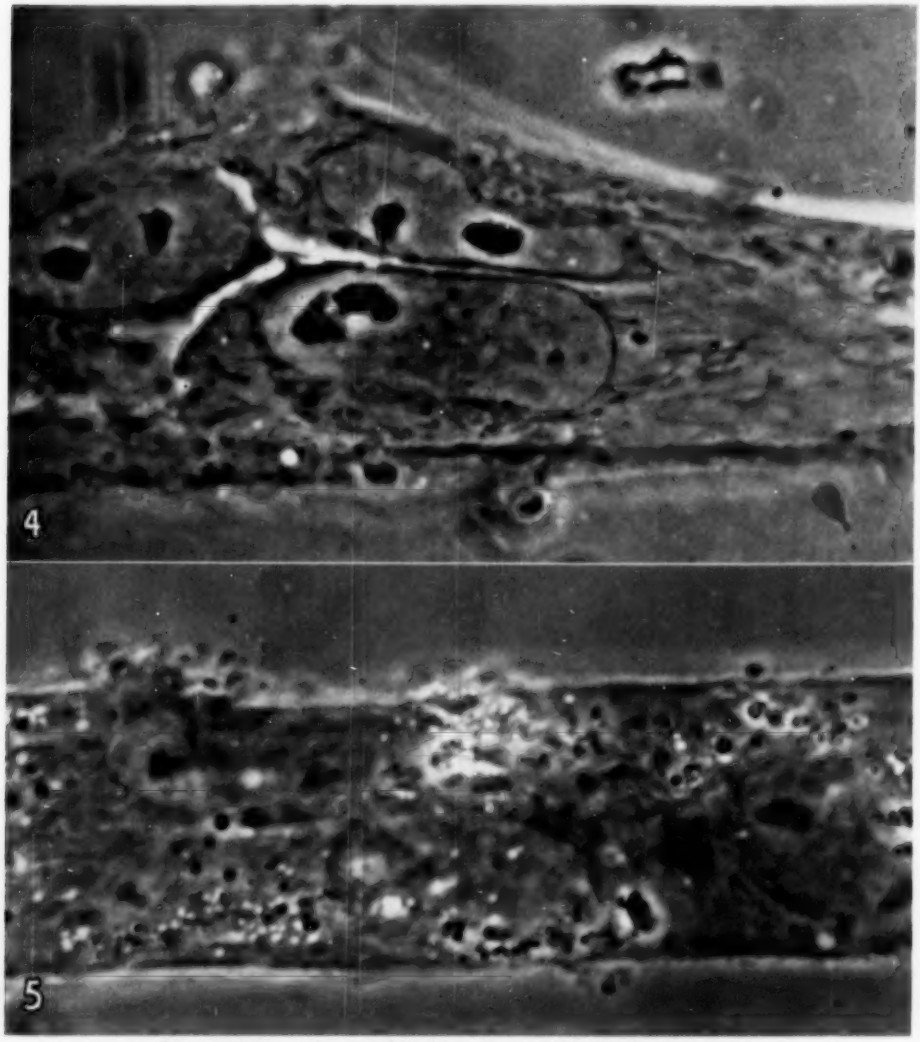


Fig. 4—Spleen culture endothelium from uninoculated pig. x 2310.

Fig. 5—Spleen culture endothelium from inoculated pig showing marked granularity, vacuolization, and irregularity of cell margins. In cinematographic records, the vacuolization and irregularity of cell margins might appear to be bubbling like a boiling heavy liquid. x 2310.

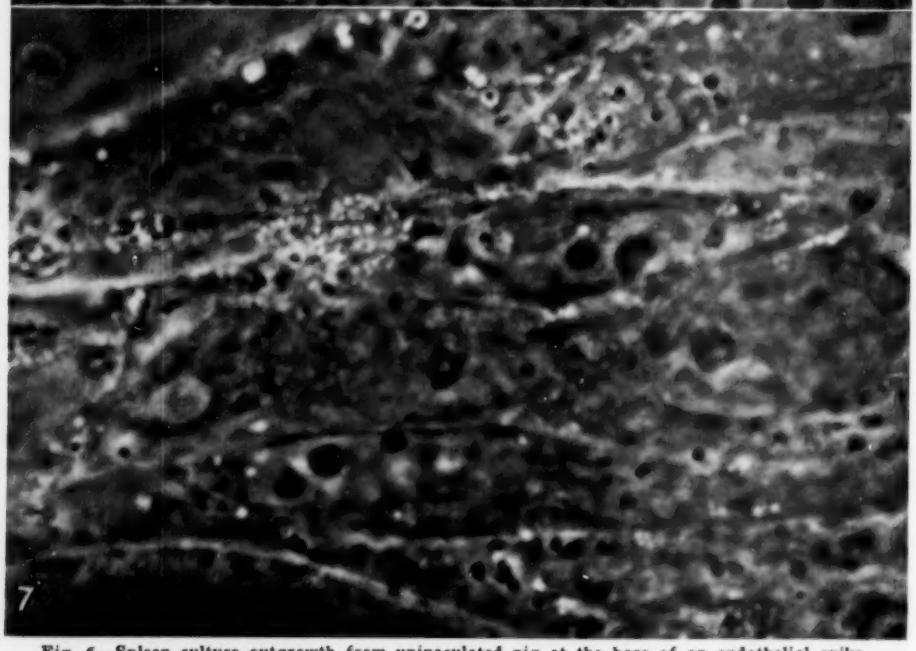
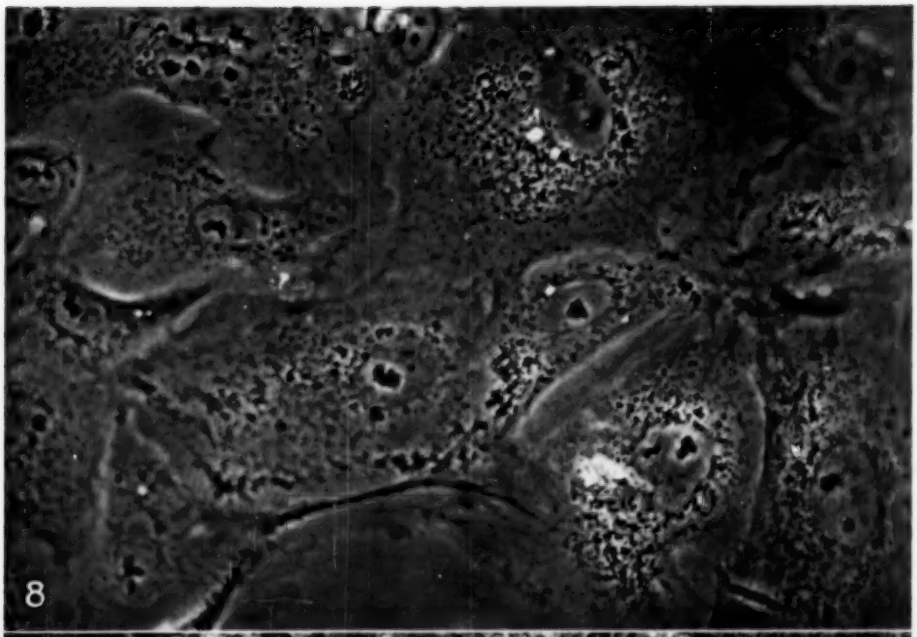


Fig. 6—Spleen culture outgrowth from uninoculated pig at the base of an endothelial spike. x 1955.

Fig. 7—Spleen culture outgrowth from an area comparable to that in figure 6 showing marked granularity, vacuolization, and irregularity of cell margins. In cinematographic records, the vacuolization and irregularity of cell margins might appear to be bubbling like a boiling heavy liquid. x 2279.



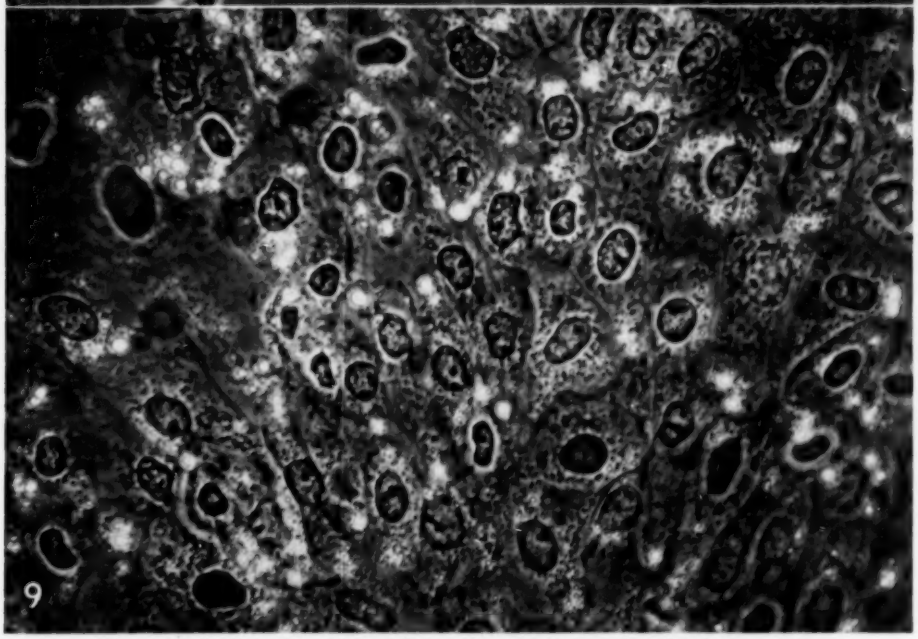


Fig. 8—Epithelial cells of lung culture outgrowth from uninoculated pig. x 493.

Fig. 9—Epithelial cells of lung culture outgrowth from inoculated pig showing diminished cytoplasm and dead or dying cells. x 493.

of cultures of cerebellum, wall of the third ventricle, corpus callosum, pituitary, and bone marrow because they appeared so similar in color. Further readings did not seem useful as the general similarity between those to be compared prevailed until the tissues were all fixed at 21 days postculture.

TABLE 1—Temperatures of Pigs on Days Following Inoculation of 2 with Hog Cholera Virus

Day	Uninoculated pig	Inoculated pigs	
		Virus in whole blood	Virus in amnionic fluid
1	101.5	102.0	101.8
2	101.5	103.5	103.0
3	101.8	104.2	104.0
4	101.8	105.2	105.2
5	102.0	105.4	105.6
6	102.2	106.4	106.8

With the use of phase microscopy, differences between the spleen culture from the uninoculated pig and that of the inoculated pig were seen readily at three days postculture. The cells which were studied were spindle cells arranged in such configuration as to suggest endothelium. These cells projected from the explanted tissue in the form of a spike or strand. The differences were manifested in the cytoplasm as shown in figures 1 to 7. There was markedly more granularity and vacuolization in the cultures from the inoculated pig. The margins of the endothelial cells in the outgrowth were somewhat rough and irregular as a result of the increased granularity and vacuolization in the cultures from the inoculated pig as contrasted with the smooth appearance of comparable areas in the cultures from the uninoculated pig.

Phase time-lapse cinematographic records of similar spleen cultures taken on the sixth day postculture showed markedly more lymphocytes moving over the outgrowth in the control culture than in the culture from the inoculated pig. The granularity of the cultures from the inoculated pig was increased in the motion pictures and seemed to be partially due to shortening and thickening of mitochondria which appear as opaque granules as their terminations bob in and out of the plane of focus.

Photographic records of lung cultures taken at five days postculture revealed a striking difference between the cultures from the inoculated pig and the uninoculated pig as shown in figures 8 to 13. The inoculated pig cultures showed a reduced amount of cytoplasm, reduced nuclear size, increased vacuolization, and death of some cells as compared to similar cells in cultures from the uninoculated pig.

Phase time-lapse cinematographic records of similar cultures taken on the sixth and nineteenth days showed no discernible difference between the cultures from the inoculated and uninoculated pigs with the exception that one field of lung epithelium at the nineteenth day showed no movement; this field was in the culture from the inoculated pig.

No differences were apparent between the fixed and the stained preparations of the cultures from all tissues. Fixation and staining caused such distortions of cytoplasmic elements as to render the cytoplasm homogenous and the nucleoli to become fragmented or wizened.

DISCUSSION

Evidence has been presented to show that tissue cultures from the spleen and lungs of pigs six days following inoculation with hog cholera virus show pathological changes which appear to be associated with the presence of the virus in those tissues.

Although observations of the tissue cultures in ordinary light conditions under low power gave the impression that those from the uninoculated pig were generally more luxuriant in amount of outgrowth, the change in indicator color was so slight that metabolic inhibition by virus through this means was not detectable.

The changes in spleen culture spindle cells and lung epithelial cells observed under phase microscopy were not readily dis-

TABLE 2—Hydrogen Ion Concentration of Tissue Cultures as Determined by Color Comparisons of Phenol Red Containing Cultures with LaMotte Phenol Red Standards at Three Days Postculture

Tissue cultured	Uninoculated pig		Inoculated pig	
	Average of 10	Range	Average of 10	Range
Corpus callosum	—	—	7.36	7.1–7.8
Liver	7.49	7.2–7.8	7.46	7.4–7.6
Thymus	7.24	7.0–7.4	7.10	7.0–7.2
Heart	7.69	7.5–7.8	7.61	7.4–7.8
Lung	7.17	7.0–7.2	7.14	7.0–7.4
Spleen	6.95	6.8–7.0	7.10	7.0–7.2

— Indicates readings were not taken because the colors were virtually identical in the cultures from the 2 pigs.

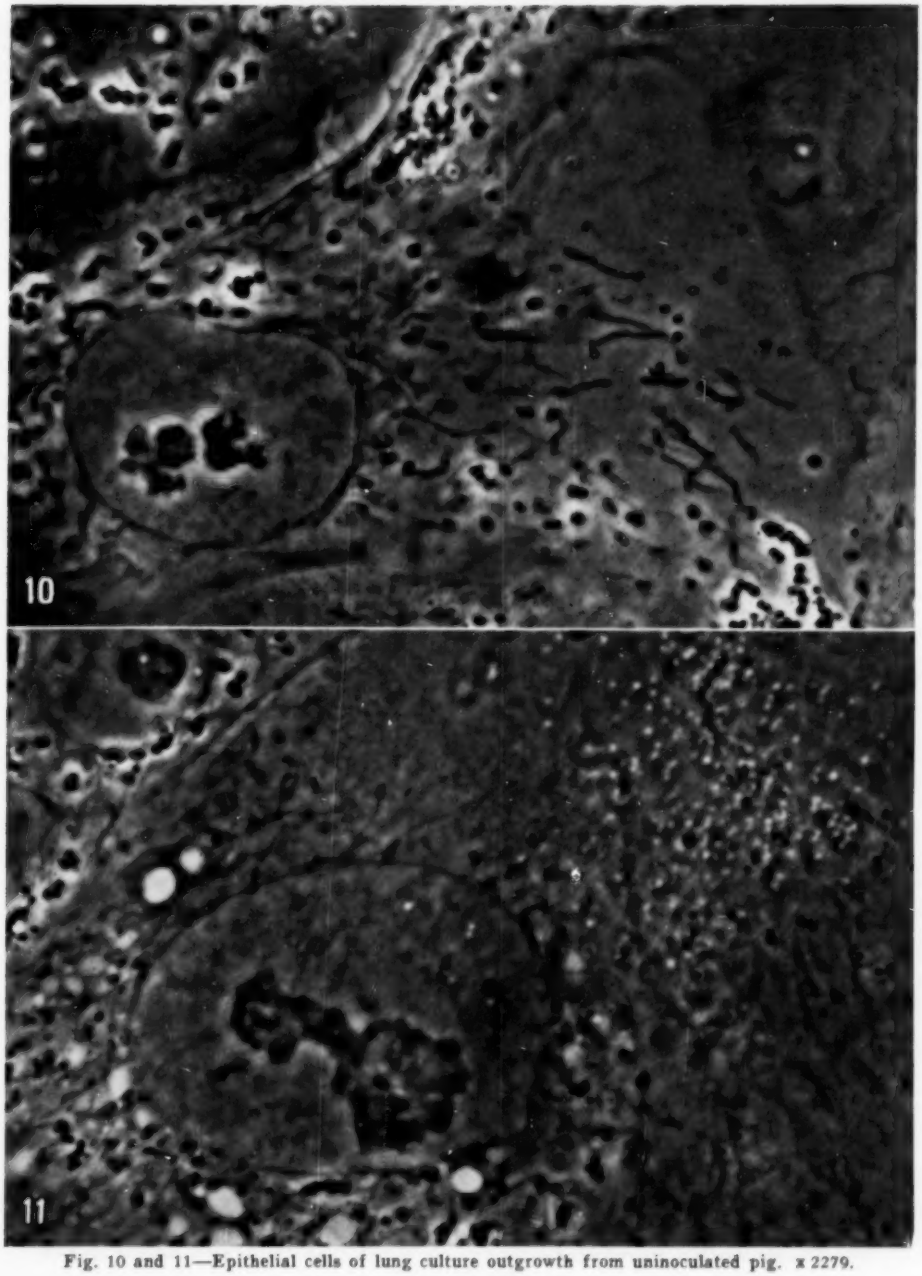


Fig. 10 and 11—Epithelial cells of lung culture outgrowth from uninoculated pig. x 2279.

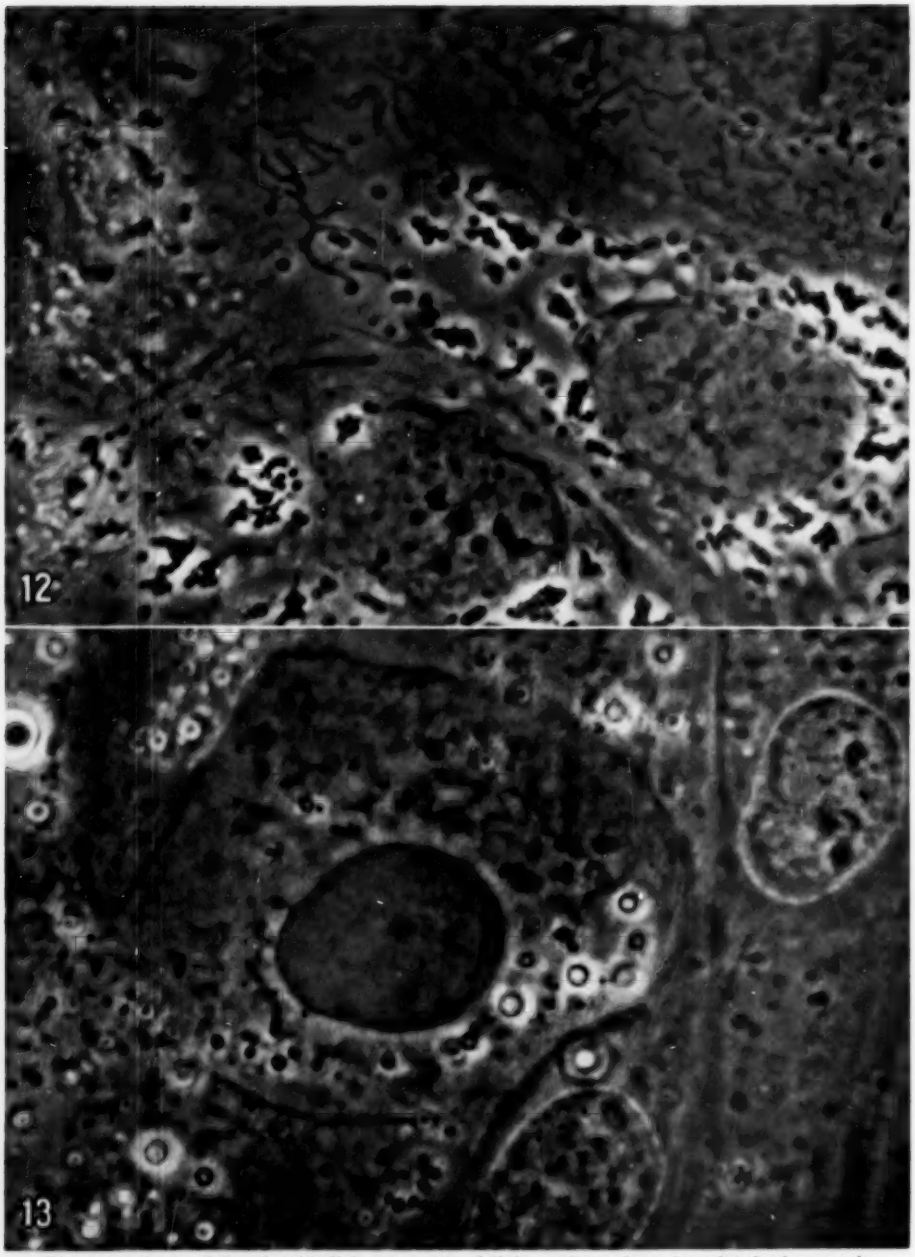


Fig. 12 and 13—Cells of epithelial outgrowth of living culture from inoculated pig seen in figure 9. x 2279.

cernible under ordinary light conditions through the wall of the culture tubes. Despite the apparent damage to the cells (fig. 3, 5, and 7) daily observations of the cultures gave rise to the conclusion that culture development and longevity were not materially affected by the presence of the virus. All the tissues cultured were not subjected to as detailed observations as was desired; therefore, it seems likely that other tissues among those cultured might well show promise on the type of examination which aroused interest in spleen and lung cultures.

The cinematographic records of the spleen cultures supported the observations made with still photography. The motion pictures permitted observation of what might well be a critical difference not noticeable under conditions of still pictures, namely, the lack of wandering lymphocytes in the cultures from the inoculated pig as compared with the uninoculated pig. This is in accord with observations of the disease in intact animals and may be of some significance in the development of the changes in lymphatic tissue.

The Jacobson whole culture staining technique rendered the cytoplasm homogenous and the nucleoli small and often fragmented; consequently, the staining preparations were useless for observations of cytopathology in the cytoplasm.

The results of these studies may be useful in the search for a laboratory means of detecting the presence of hog cholera virus.

SUMMARY

Comparisons have been made between tissue cultures of ten tissues involving 600 explants prepared from a pig six days following inoculation with hog cholera virus and of similar cultures from a susceptible litter mate. The following observations were made:

1) Significant changes were observed in endothelial cells of spleen cultures from inoculated pigs. There was increased granularity and vacuolization of the cytoplasm.

2) Time-lapse phase cinematographic records showed markedly fewer lymphocytes to be present in the cultures of spleen from the inoculated pig.

3) In epithelial cells of lung cultures from the inoculated pig there was some death of cells, reduced cytoplasm with increased vacuolization, and reduced nuclear size; however, cinematographic records of comparable cultures did not bear out these observations.

4) Hydrogen ion concentrations as estimated by indicator comparisons revealed little difference between cultures from the inoculated pig and the uninoculated pig up to 21 days.

5) The virus presumed to be present in the tissues at the time of culture apparently had only a relatively mild inhibiting effect on the gross growth characteristics of the cultures from the inoculated pig.

6) Fixation and staining techniques suggested by Jacobson caused such alterations of the cytoplasmic elements and nucleoli that these preparations were useless for further study of cytopathogenic effects of the virus of hog cholera.

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Some Pathological Effects of Anterior Pituitary Growth Hormone Preparations on Swine

L. P. DOYLE, D.V.M., Ph.D.; E. J. TURMAN, Ph.D.; F. N. ANDREWS, Ph.D.

Lafayette, Indiana

THE ASSOCIATION of the growth processes with the anterior pituitary gland has been known for at least half a century. In man, overgrowth of the skeleton in the adult (acromegaly) and extreme skeletal development in adolescence (gigantism) were known to accompany hypertrophy of the anterior hypophysis, and certain types of dwarfism were associated with decreased pituitary activity. Following the development of satisfactory techniques of hypophysectomy and methods for the purification of the growth hormone, rapid progress was made both with respect to the nature of the growth process and the role of the growth hormone in growth and other metabolic functions.

Li and Evans[6] isolated a protein from bovine pituitaries which behaved as a single substance electrophoretically and had potent growth-promoting properties without evidence of adrenocorticotropic, thyrotropic, gonadotropic, or lactogenic effects. Fishman *et al.*[5] and Wilhelmi *et al.*[8] developed methods for the preparation of crystalline growth hormone preparations which were electrophoretically and ultracentrifugally homogenous but which had minute amounts of adrenocorticotropic, thyrotropic, gonadotropic, and lactogenic activity. The yield obtained by their methods greatly exceeded that of Li and Evans.

Because of the unusual if not unique pattern of growth in swine, the initiation of marked fat deposition at the expense of muscular development by 5 or 6 months of age, it was decided to study the effects of the growth hormone in this species. As reported elsewhere, by Turman and Andrews,[7] pigs injected daily with three different growth hormone preparations gained at a slightly but not significantly faster rate. The treated pigs consumed significantly less feed per day and required significantly less feed per unit of gain than did noninjected pigs. Chemical analyses of the boned carcass revealed significantly more protein and moisture and less fat in the hormone-injected pigs. There was a significant increase in blood glucose, a slight increase in blood inorganic phosphorus, and a slight decrease in blood nonprotein nitrogen.

However, there was a great difference in the individual response of swine to the growth hormone preparations. Some animals showed no clinical or pathological evidences of an unfavorable response. Four of 11 growth hormone-treated pigs died during the course of the experiments. The pathological effects observed are summarized in this paper.

MATERIALS AND METHODS

Three trials were conducted. Barrows weighing 100 to 115 lb. were individually fed a complete mixed ration containing 16 per cent crude protein until they weighed 150 lb. and 14 per cent protein until slaughtered at 220 lb.[7] The purified growth hormone preparations were prepared by the method of Wilhelmi *et al.*[8] The hormone was dissolved in sterile saline solution at the time of injection and administered intramuscularly each day. The daily doses of the growth hormone are expressed in milligrams of growth hormone in terms of Armour standard preparation 22KR. Three lots of growth hormone were used: K45408R contained approximately 0.5 U.S.P.-T8H (thyrotropic stimulating hormone) units per milligram in addition to the purified growth hormone. Lot GH-3 contained 0.3 U.S.P.-T8H units per milligram and lot R49102¾ had a thyrotropic contamination of approximately 0.5 U.S.P-T8H units per milligram.

Suitable aseptic precautions were taken to minimize dangers of infection and in trials 2 and 3 the control animals were injected daily with sterile saline. Necropsies were done immediately following death and the histopathological examination included heart, liver, lungs, kidney, thyroid, adrenal, and anterior hypophysis.

In the third trial, the pigs receiving growth hormone exhibited arthritic symptoms late in the experiment and x-rays of the affected joints were taken. In this trial, sections of kidney, liver, pancreas, thyroid, and pituitary were obtained from both the control and growth hormone-treated animals at the time of slaughter.

RESULTS

Trial 1.—Two barrows weighing approximately 115 lb. each were injected with preparation K45408R daily until they weighed approximately 230 lb. One received 10 mg. of purified growth hormone

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per 15 kg. of body weight daily and the other 5.0 mg. per 15 kg. The hormone-injected pigs made excellent gains, an average of 1.96 lb. per day. They required less feed per unit of gain than the controls and there was a marked increase in protein and a decrease in fat in the treated swine. The protein and fat content of the edible carcass of the controls was 10.9 per cent and 49.8 per cent, respectively, and the average of the hormone-treated pigs was 14 per cent and 35 per cent. The growth hormone-treated pigs showed no detected evidence of an unfavorable response at any time during the trial.

Trial 2.—In this trial, 7 barrows weighing approximately 110 lb. were used. Three served as controls and were injected daily with sterile saline. Two pigs received growth hormone (lot GH-3) at a level of 4.5 mg. per 15 kg. of body weight and 2 received 2.25 mg. per 15 kg. These dosages were less than half those used in the first trial. During the first 27 days, all pigs gained rapidly. On the thirtieth day of treatment pig 1, receiving 4.5 mg. per 15 kg., decreased its feed intake from 7.25 lb. per day to 5.29 lb. and on the thirty-fourth day refused feed. The pig was extremely nervous, exhibited muscular trembling, and, when forced to move about, showed clonic convulsive contractions of the muscles. The animal did not eat and death followed a severe convulsive seizure on the thirty-ninth day of the experiment. At necropsy, the kidneys of pig 1 were found to have pale cortices and cloudy pelves. The liver was enlarged and there were some adhesions to the diaphragm. The heart was enlarged, the left atrioventricular valve thickened and edematous, lungs were edematous, and the forelegs showed subcutaneous edema. Microscopically, the kidney showed fatty and albuminous degeneration. The liver was edematous and there was vacuolation of the liver cells.

Pig 2, receiving the same level of growth hormone as pig 1, gained rapidly until the forty-first day of the experiment. At that time, it weighed 188 lb. and was gaining at a rate of 2.00 lb. per day. The animal then suddenly stopped eating, showed the same type of muscular trembling as pig 1, had intermittent clonic convulsive muscle contractions, and died on the forty-fifth day. The kidneys were enlarged and appeared fatty. Microscopic examination showed fatty and albuminous degeneration and sloughing of the tubular epithelium. There were microscopic evidences of edema of the heart, thyroid, and adrenal capsule. There was some postmortem degeneration of the adrenal and thyroid. The thyroid follicles were without stored colloid, and the epithelium was hypertrophied and markedly dissociated. The liver was enlarged and appeared fatty. One lung appeared normal and the other showed evidence of hypostatic pneumonia.

The 2 pigs which received growth hormone at a level of 2.25 mg. per 15 kg. of live weight gained at the rate of 2.56 lb. per day throughout the experiment in contrast to 2.32 lb. for the controls. As in trial 1, they consumed slightly less feed per day, required less feed per unit of gain, and stored considerably more nitrogen than the controls. The composition of the control carcasses was 11.0 per cent protein and 48.3 per cent fat and of the hormone-treated pigs 13.0 per cent protein and 38.8 per cent fat.

No unfavorable effects of lot GH-3 growth hormone were observed in the pigs which received it at a level of 2.25 mg. per 15 kg. of live weight.

Trial 3.—Fortunately, sufficient amounts of GH-3 were available to allow its use at a level of 4.5 mg. per 15 kg. in 1 pig during trial 3. A new lot of hormone (R491024) was administered to 2 pigs at a daily level of 6 mg. per 15 kg. and to 2 pigs at a rate of 3 mg. per 15 kg. of live weight.

The pigs which received the hormone at the 3-mg. level and the pig injected with GH-3 at the 4.5-mg. level gained at a rate of 2.54 lb. per day and 2.04 lb. per day, respectively, in comparison with the controls which gained 2.11 lb. per day. As in the other trials, the pigs which received growth hormone throughout the experiment consumed less feed per day, required less feed per unit of gain, and contained more protein and less fat than the controls.

Pig 23, injected with growth hormone at the 6-mg. level, showed a decrease in appetite on the seventh day of treatment and was extremely nervous. At the end of 14 days all food was refused, the animal exhibited continuous muscular trembling and clonic contractions when forced to move. Death occurred on the seventeenth day of treatment during a convulsive seizure. At necropsy, the muscular tissues were ex-

tremely pale and generalized edema was apparent. The heart was atonic, the mitral valves thickened, there was an increase in pericardial fluid and edema beneath the endocardium. The liver appeared grossly fatty with evidences of localized parenchymatous degeneration. Microscopically, there was extensive vacuolation of the liver cells and some widening of the sinusoidal spaces. No evidence of any inflammatory or infectious change in the liver was observed. The kidneys were pale and there was marked fatty degeneration with some albuminous degeneration. The stroma of the pancreas was edematous and the islet cells had undergone granular degeneration and had largely lost their outline. The epithelium in some pancreatic ducts was loosened and desquamated. The adrenal capsule was edematous but the glomerulosa and fasciculata appeared to be within the normal width range for swine. The reticularis appeared to be somewhat disorganized. The thyroid epithelium showed both hypertrophy and hyperplasia, little colloid was present, and the interstitial tissue was edematous.

Pig 25, which also received the 6-mg. level of growth hormone, exhibited clinical signs almost identical with those of pig 23. On the eleventh day of injection, he appeared to be following the same course so hormone treatment was discontinued for 24 days. Within ten days after cessation of treatment, feed consumption and rate of gain returned to normal levels. Hormone injections were resumed and at the end of three weeks the pig was in a moribund condition and was necropsied. Extreme ascites, hydrothorax, hydropericardium and subserosal edema were observed. Grossly, the liver appeared to be completely infiltrated with fat. The bile duct was patent and bile was entering the duodenum. The heart and lungs were grossly normal although the heart fat appeared slightly icteric. Microscopically there was vacuolation of nearly all of the liver cells, which was assumed to be the result of fatty degeneration. Some of the liver cells, particularly those near the central vein of each lobule, showed granular degeneration or cloudy swelling. In some liver cells the nuclei were indistinct, in some the nuclei were resting against the cell wall. Some nuclei were pyknotic and some showed karyolysis.

The kidneys showed extensive fatty degeneration. There were varying degrees of nuclear degeneration and some albuminous degeneration. The vascular tufts of some glomeruli appeared shrunken. There was no microscopic evidence of any inflammatory processes prior to the degenerative changes which occurred.

Some edema of the pleura and atelectasis in some areas of the lungs were found but no evidences of inflammation. The heart muscle was edematous and showed evidence of early albuminous degeneration. The mesenteric lymph nodes were grossly enlarged and contained polymorphonuclear leukocytes and giant cells. In no other tissue examined were evidences of any inflammatory process found.

In view of the extreme ascites, edema of the adrenal capsule was not unexpected. The adrenal glomerulosa and reticularis appeared normal, but there was evidence of dissociation in the reticularis. The thyroid histology was within the normal range although the epithelium was slightly hypertrophied. There was passive congestion of the spleen and a reduction in splenic pulp in some areas.

Pig 27, which received growth hormone at the level of 3 mg. per 15 kg. of live weight, exhibited stiffness and soreness of the legs and swelling at the hocks and knees at the very end of the trial. Since the symptoms were suggestive of rheumatoid arthritis and since they appeared to a lesser degree in the other growth hormone-treated pigs, it was decided to x-ray the limbs of both treated and control pigs. Pig 27 had a temperature of 104 F. but no evidence of arthritis was found on x-ray examination or at necropsy and no bacterial growth was obtained in synovial fluid cultures. Histopathological examinations of the liver, lungs, adrenal, thyroid, pancreas, and heart showed them to be essentially normal with some slight edema. The tubular epithelium of the kidney showed some albuminous degeneration.

Pig 21, which also received the 3-mg. level of hormone, showed some clinical evidence of lameness and soreness of the joints. At slaughter, there was no evidence of joint capsule involvement. The liver and thyroid were normal. Some granular material was observed in the kidney tubules.

Pig 26, which received 4.5 mg. of GH-3 per 15 kg. of body weight, as did the pigs

in the second trial, both of which died, showed some lameness but no evidence of arthritis on x-ray examination. The liver, pancreas, and thyroid were normal histologically. Although the kidney contained some areas of vacuolation and albuminous degeneration, it was considered functionally adequate.

Kidney, liver, pancreas, and thyroid were obtained from the saline-injected controls at slaughter. Both of these pigs showed some soreness and lameness. This was more or less true in all the animals maintained on concrete floors in the three trials and in other studies in which pigs were kept in individual pens for long periods. However, x-ray examination of the controls did not reveal arthritic changes in any of the limbs. No definite pathological changes were found.

Discussion

As previously summarized by Turman and Andrews,[7] it is obvious that the administration of three different lots of purified growth hormone did produce marked alteration of the normal growth pattern of swine. Although growth rate was not significantly increased, the efficiency of feed utilization was improved and there was an increase in the protein content of the animal's edible carcass of approximately 30 per cent. There was an immediate increase in blood glucose and some reduction in blood nonprotein nitrogen.

In the animals which survived, it seems reasonable to conclude that the over-all effect was beneficial when measured by usual standards. It is further suggested that swine are capable of significant improvement in lean-fat relationships. However when the response was unfavorable, it was sufficiently disastrous to be described as lethal.

The extreme difference in response can not be satisfactorily resolved with the data available. The nature of the difference, however, should permit a certain amount of speculation.

The most obvious difference lies in the three lots of growth hormone used. Although great care is exercised in the preparation and assay of these, or any preparations of proteinaceous hormones, they can at best be described as only relatively pure. However, one of the preparations which produced death at the level of 4.5 mg. per 15 kg. of live weight in trial 2 was without ill effects in trial 3.

Differences in dosage levels do seem to be related to unfavorable effects. The deaths were confined to the higher dosage levels in both trials 2 and 3. However, the levels used in both pigs in trial 1 were greater than those which produced death in trial 2, and the response in trial 3 with GH-3 was unlike that in trial 2.

In spite of the small numbers of animals involved, it is tempting to speculate that individual variability of the swine is involved. It is felt that the results in this study support in general the views of Baird *et al.*[1] that growth stasis at maturity is the result of a relative deficiency of growth hormone. Their data indicated that the anterior pituitary secretes a constant amount of growth hormone at all ages; but the pituitary does not increase in size proportionately with increasing body weight. Thus the animal is subjected to gradually decreasing amounts of growth hormone per unit of tissue as growth occurs. This study indicates that swine can respond to increased levels of growth hormone and do deposit increased amounts of protein, at least until weights of 220 lb. are attained. If it is true that there are wide individual differences in response to the growth hormone in swine, these differences might be utilized in the selection of swine for improved meat-type. The unfavorable response of some swine to the growth hormone appears to involve the liver and kidneys after a very short time. Purified growth hormone preparations have been shown to have a diabetogenic effect in several species, the dog,[2] the cat,[4] and man.[3] The preparations used in trial 3 produced a marked increase in blood glucose within one week. Since the data are too scant to evaluate the diabetogenic effects in swine, further studies on this point are highly desirable.

Summary

Three preparations of purified growth hormone were administered to swine beginning at 100 to 115 lb. in weight and continuing until approximately 220 lb.

Four of 11 animals treated with growth hormone died during the experiments.

In animals in which no unfavorable response occurred there was a slight increase in growth rate, an increase in feed effi-

ciency, an increase in protein, and a decrease in fat in the edible carcass.

In animals which did not tolerate the growth hormone preparations, death occurred in from 17 to 45 days. The clinical signs were severe muscular trembling and clonic convulsions terminating in death. The most common histopathological changes were fatty infiltration and degeneration of the liver, fatty and albuminous degeneration of the kidney, and generalized edema. There was no evidence of any infectious or inflammatory process.

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Exudative Epidermitis of Pigs

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ALMOST 50 years ago, Melvin [42] wrote:

> Affections of the skin in hogs are very common; and as some of the skin lesions are frequently associated with infectious diseases, a thorough knowledge of the various cutaneous affections is of great importance from the standpoint of a differential diagnosis.

Excepting parasitism, viral exanthemata, swine erysipelas, and photosensitization, little has been accomplished in fulfilling the needed differentiation referred to. Malkmus [37] believed the condition of the skin indicated the state of health—"If the condition of the hair coat is bad, notwithstanding good care and shelter, it may be assumed that the animal is suffering from ill health." Cipollaro and Schwartz [8] felt that skin diseases, with few exceptions, are an expression of derangement of the normal physiology of the organism—"Many pathologic states—metabolic, endocrine, infectious or malignant—frequently manifest themselves first in the skin."

During the past few years, a skin disease of suckling pigs has been reported with increasing frequency in Iowa. Names employed in reports include "pustular dermatitis," "greasy pig," "necrotic dermatitis," "infectious dermatitis," "exfoliative dermatitis," and "exudative dermatitis." Among Middlewest swine, the incidence of the disease seems to be increasing. In some Iowa droves, it has caused serious losses. All of our basic information has been gleaned from reports of studies made by continental workers. A great deal of information is still lacking concerning details of this disease including predisposition, etiological factors, pathogenesis, signs, and lesions. It is uncertain whether this is a specific disease or a manifestation held in common by several diseases.

REVIEW OF LITERATURE

Spinola [47] was the first to publish on the subject. He used the term "hautauschlag," which means skin rash or "(morbi efflorescentiae cutanae)." He reported that it occurred rarely in swine and that, while it was recognized as an entity, it could also appear with other diseases such as swinepox, foot-and-mouth disease, and mange. The uncomplicated form was seldom found. When seen in nursing and weanling pigs, it was called a baby pig skin rash. This rash first made its appearance as a formation of small vesicles that occurred in dense clusters on various parts of the body, especially around the eyes. These vesicles soon ruptured and formed a very superficial skin ulcer covered with a brown scab. These lesions seemed to spread and coalesce until the entire body was involved. The eyelids were swollen due to edema, their opening narrowed, and the edges gummed with a suppurative exudate. The tongue was usually coated. Diarrhea might appear, but constipation was more common. Usually anorexia and salivation preceded the appearance of the skin rash. The early symptoms usually disappeared as soon as the skin lesions appeared. According to Spinola, the cause was attributed to the "rich concentrated ration of the sow or the weaned pigs." He also mentioned having observed a gastritis, the significance of which he did not explain.

In his "History of Animal Plagues of North America," Bierer [6] called attention to the great losses of swine (from numerous causes) in 1869 and at regular intervals thereafter. There was no reference to dermatitis of swine in his report for the period 1732 to 1915.

Roadhouse and Hayes [52] probably were the first in North America to recognize this disease. They described several skin diseases, including necrotic dermatitis, phthiriasis, swinepox, erysipelas, and sarcoptic mange, but the details of their report were not clear enough to make an accurate comparison.

The lesions of this disease, described by Hieronymi,[28] do not agree with those of other authors. Thin scales of exfoliation were found on the skin. These scales appeared in many stratified layers, lying loosely on skin which was not much inflamed. Histologically, only a few changes were seen. There was a slight edema of the stratum spinosum in which the mosaic pattern was slightly disassociated or distorted. In some places the stratum corneum was elevated by edema; no vesicles were formed, but a flatly elevated lesion showed a slight parakeratosis. The connective tissue of the corium and of the skin glands was unchanged, and slight vasodilation was observed. Scratching accentuated these changes.

Almost all of the pigs affected by this disease, according to Dettweiler *et al.*,[11] were young, neglected, weak, anemic, and ill with other diseases. Mange and other forms of dermatitis were often

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Taken from "Clinical and Pathologic Studies of a Dermatitis of Immature Iowa Swine," M.S. Thesis, Library, Iowa State College, 1955.

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mistaken for this disease which they termed "russ der ferkel" or "ferkelausschlag." They observed that the skin was first grey and lusterless; then the hair and skin, especially of the shanks, chest, belly, eyelids, ears, and often all over the body, became covered with a sticky fatty coating. This coating was an accumulation of sebum, which decomposed and turned black. Because of this glandular involvement, it was also called seborrhea. The vesicles that formed at this stage soon ruptured to form ulcers over which a black pitchlike scab soon formed. In a more chronic form, the skin was thickened and wrinkled and the lesions might have been confused with swinepox or mange.

Transmission experiments were conducted by Klobuk.[69] He found it to be a specific infectious disease, easily transmitted artificially, with recovery resulting in acquired immunity. He successfully transmitted the infection by inoculations with a filtrate prepared from an emulsion of the skin of affected animals. He proposed the name of "exanthema suis exfolians sive exfoliativa," and that the causal agent "probably is a virus." The details of his work were not available.

Troxler[92] accumulated data from studies of cooperative swine-raising herdbook records to furnish evidence to support his theory of a congenital predisposition to the disease. This predisposition was traced back to the sixth generation on both paternal and maternal sides. These parent pigs were known to have had as many as three separate attacks, each followed by recovery. His report also included results of studies and experiments with cases encountered in his private practice. The occurrence of the condition was nonseasonal. Pigs were found to be susceptible any time after they were 3 weeks old. Pruritus was observed in all of his patients. Various diets, ration supplements, and medicaments were employed with more or less satisfactory response. Success consistently followed the application of therapy for sarcoptic mange. In summary, he recorded that "russ of piglets" was a disease often beginning with hemoglobinemia, diarrhea, and stunting.

In addition to the preceding information, reports of this disease have been made from Japan by Futamura,[16] from Germany by Schnyder,[84] Mussemeier and Hofferber,[66] and Glässer *et al.*,[17] from Czechoslovakia by Pekar,[68] and from the British Isles by Mayall,[60] and by Luke and Gordon.[54] In the United States, other reports have been made by Lynch,[55] White,[95,96] Kinsley,[26] Kernkamp,[27] and Gellen.[15] According to Breed,[4] Moore,[64] Quin,[69] Spear,[89] and many others, this disease has been discussed at veterinary association meetings for the last 20 years.

MATERIALS AND METHODS

General Procedures.—The pigs used for this study were procured through the facilities of the Iowa Veterinary Diagnostic Laboratory. Selection of the material for study was limited to farm-farrowed pigs without apparent complicating conditions. A report form was employed for collecting data in addition to the record card routinely used for all cases submitted to the laboratory. In all, the data and materials collected for this study were from 20 droves, comprising 374 litters, and totalling 3,055 pigs. Over 40 necropsies were performed.

Immediately following electrocution of the pigs, pieces of skin, approximately 2 cm. by 6 cm. were removed from various parts of the body for histopathological and other studies. During a routine necropsy, the following other tissues were collected for histopathological studies: kidney, liver, spleen, and the prescapular, prefemoral, and superficial inguinal lymph nodes. Routine bacteriological studies were made of these and of the heart's blood. The inoculum was streaked on blood-agar plates and incubated aerobically at 37 C. The plates were discarded if negative after an observation period of 48 hours.

The tissues collected for the histopathological studies were taken from 24 pigs representing 17 of the 20 droves studied during the period from July 3, 1953, to April 22, 1955. This included 63 lymph nodes, and sections from 18 livers, 17 kidneys, two spleens, and 101 pieces of skin.

Tissue Techniques.—Modifications introduced during the course of the work resulted in handling the skin specimens along the lines described by Cowdry[9] as follows. A sharp knife was used in removing the pieces of skin. As far as possible, these pieces were cut so that the direction of the hair was parallel with either a side or end of the piece. The hair was trimmed with scissors and the specimen wetted with tap water. The piece was then placed, with the epidermis down, on a wooden tongue depressor and held in position with common pins placed at frequent intervals along the margins. The tongue depressor was weighted with pieces of lead and the specimen then placed into a 10 per cent solution of formalin in 0.85 per cent sodium chloride solution buffered with calcium carbonate. After four hours, the skin was removed from the board, trimmed with a sharp wet surgical knife and replaced in the fixative with the epidermis down. After 20 hours, the pieces were placed in a 70 per cent solution of ethyl alcohol for storage or further processing.

Usually after 24 hours in the 70 per cent alcohol, the pieces of skin were removed for study with the aid of a binocular dissecting microscope. Then about one-third of a piece of the skin was cut off and the remainder returned to the alcohol for storage. The smaller piece was marked for sectioning and the hair closely clipped with fine dissecting scissors. Then the specimen was consecutively placed in: 95 per cent ethyl alcohol for 24 hours; absolute ethyl alcohol for eight hours; chloroform for 12 hours; chloroform-Altman's mixture (paraffin, beeswax, and stearin) kept liquid at 56 C. for four hours; and finally embedded in Altman's mixture. The other tissues were similarly fixed and embedded. All skin sections were cut between 7 μ and 10 μ thick. Sections of all other tissues were cut 7 μ thick. All sections were fixed onto slides with Mayer's albumen, then

stained, and finally mounted in synthetic resin mounting medium.

Staining Methods.—All tissues were stained with Delafields hematoxylin and counterstained with ethyl eosin.

In addition, sections of 51 lymph nodes and 89 pieces of skin (representing 20 of the 24 pigs and 15 of the 17 droves) were stained by a modification of the periodic acid-Schiff method (Hotchkiss-McManus stain) as discussed by Kligman *et al.*,[20] McManus,[41] and Gomeri.[18] The Schiff's reagent was prepared according to the methods described by McManus,[41] Lillie,[32] and Pillsbury and Kligman.[47]

Controls.—With each series of sections stained by the periodic acid-Schiff method, at least one section of tissue, known to contain the organisms of aspergillosis, histoplasmosis, or blastomycosis, was carried along in the process as a control.

To obtain normal skin specimens for direct comparison with the abnormal, pieces of skin from 27 different areas were taken from an apparently normal 5-week-old pig weighing 20 lb. These pieces of skin were prepared, fixed, and stained with hematoxylin and eosin in the manner already described.

The histology of the skin of swine has been described by Gurlt,[19] Ellenberger,[13] Kränzle,[21] David,[10] and Ham;[20] the lymph nodes of swine by Richter,[51] Sabin,[53] and Trautmann,[60] the liver of the pig by Mall,[38] and reconsidered by Elias and Lazarowitz;[12] the kidney of the pig by Tereg,[55] and Trautmann and Fiebiger.[61] The cytology of the spleen has been described by Jackson and De Boom.[25] These descriptions were used as a basis for determining the presence of any abnormalities found in the various tissues examined in this study.

RESULTS

Definition.—The disease, as a result of observations made in this study, is defined as follows: An acute, generalized dermatitis involving the entire body surface of young swine, characterized by sudden onset and a short course; marked by hyperhidrosis, excess sebaceous secretion, exfoliation, exudation, and without pruritus; resulting in loss of skin function, extreme dehydration, rapid exhaustion, usually terminating in death. A secondary bacterial invasion accompanies the process.

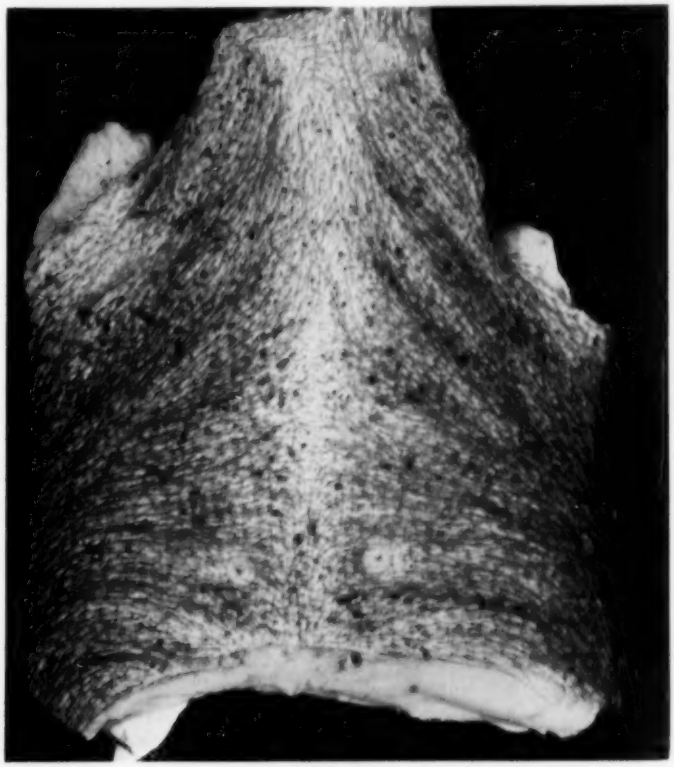

Fig. 1—Sternal area showing skin lesions typical of the early stages of exudative epidermitis of pigs. Actual size.

Etiology.—The scope of this investigation did not embrace a search for the causative agent. Unfortunately it is still unknown.

Incidence.—The disease is widespread in Iowa and neighboring states. Reports received from many sources indicate its prevalence has increased during the past six years. The disease attacks the young pig at any time between 5 and 35 days of age. The average age of the pigs studied was 21 days. The younger the pigs the lower the resistance, the shorter the course of the disease, and the higher the morbidity and mortality.

Any number of the litters on a farm may be affected. If the disease appears in a litter, all of the pigs of that litter usually, but not always, become affected. On one farm, half of each litter was affected.

Of the 3,055 pigs in the study, 881 (28.8 %) were reported as having the skin disease. Of these 881 pigs, 597 (67.8 %) were reported as having died, a total mortality of 19.5 per cent.

The mortality varies greatly. In one drove 5 of 81 pigs died, and in another, 145 of 160 pigs died.

Predisposing Factors.—No predisposing factors could be found. The occurrence was not seasonal. Variations in housing made no noticeable difference. In this study, there were no reports of the pigs having been chilled or overheated. No differences were discerned by the use of oat straw, shredded corn stalks, or ground corn cobs for bedding. In most droves, the sanitary conditions on the farm were reported as being good.

The feeding programs were considered satisfactory. Animals that had access to pasture were as much affected as those maintained on drylots. The ration of the nursing pigs usually included supplemental creep feeding. Pigs were weaned at an early age in only one drove.

There was no evidence that breed, sex, color, size of litter, or heredity influenced the occurrence of the disease. Pigs of first, second, and third litters were equally affected. Other diseases, including external parasitisms, did not appear to complicate the clinical picture.

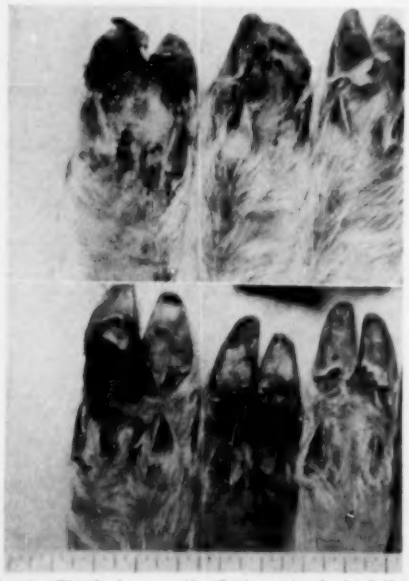

Fig. 2—The lesions on the feet are typical of those described under the peracute form of exudative epidermitis of pigs. Actual size.

Pathogenesis.—At the onset of the disease, the pig shows listlessness, eyes less bright than normal, a drawn facial expression, drooped ears and tail, dull skin, and lusterless hair. When stirred from the nest, it appears brighter for a short period.

The entire skin surface is covered with thin dry delicate scales. The color of these scales varies with the cleanliness and color of the skin. They appear grey on black skin, reddish brown or rusty on red skin, and light orange on white skin.

On the second or third day, the attitude varies from dejection and apathy to restlessness and anxiety with some anorexia and thirst. The skin is more taut and has a slightly thickened and swollen appearance. With the aid of a dissecting microscope, the polygons of the skin pattern are seen to be swollen, their bands of demarcation are more prominent, and tiny brown spots begin to appear at the base of the hairs and at the openings of the sweat glands between the hairs (fig. 1). These brown spots increase in number and size. The skin is damp and oily.

As the disease progresses, the animal becomes more languid and depressed and the anorexia increases. Constipation is usual but diarrhea may appear temporarily. There is a loss of body weight. Significant pyrexia has never been reported, the hair remains undamaged and intact, and pruritus is not observed. In spite of the great tissue damage there seems to be no pain.

The course of the disease, from this point, may follow three different patterns. For the purpose of description they are designated peracute, acute, and subacute. Transitions and variations between these forms may appear at any time.

Peracute Form.—At any time after the brown spots appear, the entire body may rapidly became covered with a moist, sticky, greasy exudate composed mainly of sebum, serum, and sweat. Cutaneous erythema is marked. This process stops abruptly and a thick line of gummy exudate forms at all the mucocutaneous junctions. The general condition becomes worse with complete anorexia, increased depression, and weakness. The production of exudate may cease temporarily and that present may dry as a thin scab coating. Upon the resumption of exudation, this scab is loosened. It can be readily removed, exposing a raw, highly inflamed skin. This reaction is more easily observed where the hair is thin. The feet were constantly affected in all pigs studied, involving both the plantar and volar interungulate skin and associated digital bulbs (fig. 2). The skin may have a strong rancid odor. There is severe emaciation, dehydration, and exhaustion. Death usually occurs in three to five days.

Acute Form.—Compared to the peracute form, the outpouring of exudate and development of the disease is less rapid but constant and the skin becomes more thickened and wrinkled. The brown spots persistently increase in number and size. Tiny vesicles and pustules develop and rupture forming ulcers. Before rupturing they may coalesce, thus undermining larger patches of epidermis. This phenomenon can not always be observed grossly. The skin remains moist and greasy and the exudate accumulates and dries (fig. 3 and 4). The large scabs and crusts become fractured along bending furrows and cleavage lines thus

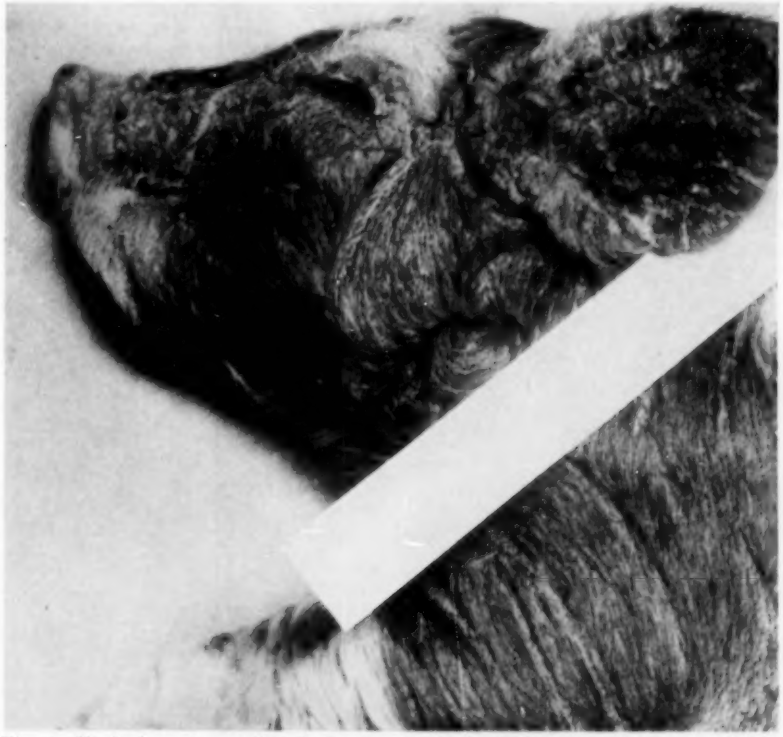

Fig. 3—Head view of a moribund pig, approximately 16 days old, showing the acute form of exudative epidermitis of pigs.

forming deep fissures (fig. 5). Anorexia is complete. Emaciation, dehydration, and weakness are progressive. Death usually occurs in four to eight days.

Subacute Form.—This form, the least commonly encountered, develops in the same manner as the acute form but the skin becomes even more thickened and wrinkled (fig. 6). The entire course may require three weeks. The mortality is lower than in the other two forms, but death may occur at any time. Recovery, when it occurs, is slow.

Gross Pathology.—Necropsy revealed marked dehydration and emaciation.

All superficial skeletal lymph nodes were enlarged and soft. Many show peripheral hemorrhages and pigmentation. On sectioning, the cut surface bulged and lymph was released. When the course had been somewhat prolonged, their texture was more firm. Occasionally miliary necrosis and abscess formation were found.

The heart was mildly dilated. The arborization of the coronary vessels was pronounced. The lungs were usually collapsed and congested. The stomach and small intestine were usually contracted and contained small amounts of mucus, bile, sour ingesta (stomach), or chyme (small intestines). The contents of the colon were usually dry and pasty. The liver was usually slightly swollen, cyanotic, and dark.

In the urinary system of every affected pig examined, a precipitate could be readily seen impacted in the papillary ducts of the kidneys (the so-called uric acid infarcts) or lying free in the renal pelvis, ureters, or urinary bladder. This duplicated the findings by Madsen *et al.*[36] who

Fig. 4—Anteroventral view of the same pig as shown in figure 3.

reported this condition to be associated with acute uremia.

No other significant gross lesions were found.

Histopathology.—Skin.—All of the pieces of skin collected were examined with a binocular dissecting microscope.

The swelling of the skin was best exemplified along the lines where it bends. It was also observed at the openings of the hair follicles where the epidermis was pushed up around the hair shaft to a level above the normal skin surface. This produced a tiny depression of the skin in the form of a cup (fig. 7) which was filled with a gummy, waxy material, with a ball-like shape, surrounding the base of the hair. This material, which varied from light yellow to dark brown, was composed mainly of sebum, mixed with cellular debris, bacteria, and dirt. As its production continued, it spread and united with its kind from neighboring hairs, forming the brown spots seen on gross examination.

The brown spots found at the openings of the sweat glands were formed in practically the same manner but were smaller and contained sweat in place of the sebum.

Under the compound microscope, skin sections taken during the early stage of the disease showed a severe active hyperemia of the dermis (fig. 8), also a marked dilatation of the lymphatic capillaries. As the disease progressed, the blood and lymph capillaries were even more dilated.

All of the sweat glands were dilated and active with none in their cyclic resting stage. The secreting epithelial cells were swollen and the cell membranes were indistinct. The nuclei were swollen, finely granular, and many cells contained two nucleoli. The increased activity of the sebaceous glands could be recognized by the cellular activity and the material contained in the lumen of their ducts (fig. 9 and 9a).

The epidermis showed slight hypertrophy, hyperplasia characterized by a lengthening of the interpapillary pegs, and a mild increase in the number of layers of cells of the stratum spinosum (fig. 10). The nuclei of the cells of the stratum granulosum were more distinct than in normal skin. The stratum lucidum could not be identified. Hyperplasia of the stratum corneum was indicated by the increase in its thickness and the nuclei it contained, even though individual cellular outline was lost.

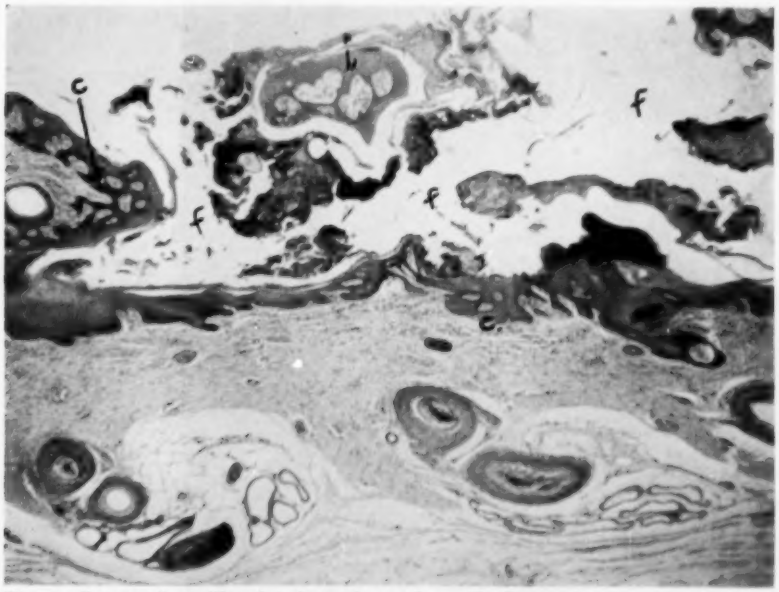

Fig. 5—Compound papillae (c) formation and a wide fissure (f) are two prominent lesions often observed in exudative epidermitis of pigs. In the upper center is an islet of skin (i) with marked hyperemia of the papillary dermis. Back area. x 35.

There was a moderate increase in the number of eosinophiles in the dermis.

Multiple foci of bacteria, having a coccoid shape and taking the hematoxylin stain, were present on the surface of the skin.

As the disease progressed, a separation of the epidermis from the dermis began, indicating edema. The cells of the stratum cylindricum were swollen and their cytoplasmic filaments less distinct. The rate of the epidermal hyperplasia increased. There were focal areas of intercellular and intracellular edema of the stratum spinosum. In these areas, the tonofibrils were less distinct. At the same time, the stratum corneum became thicker, cracked, and fissures developed in which bacteria were found. An occasional lymphocyte and neutrophil could be seen, apparently migrating upward through the epidermis. They collected in the vicinity of the bacteria forming tiny pustules in the stratum corneum.

In the areas where the intracellular edema existed, the affected cells underwent hydropic degeneration and disintegrated. Simultaneously the intercellular edema separated the cells of the epidermis and tiny vesicles were formed which enlarged and coalesced forming larger vesicles. The vesicles ruptured, otherwise neutrophils infiltrated, forming a pustule which could rupture. The vesicles and pustules could be seen any place in the upper half of the

Fig 6. — Sternal area showing skin lesions of the subacute form of exudative epidermitis of of pigs. Actual size.

epidermis. Throughout this process, there was only a very light infiltration of the dermis with leukocytes, principally eosinophils.

The dermal and epidermal inflammatory changes of the hair follicles proceeded along with that of the neighboring areas, except that pustules and vesicles were not seen. The major portion of the activity was restricted to that part of the follicle above the point where the sebaceous gland duct emptied. The accumulation of exudate from this reaction resulted in plugging of the follicle.

If the course of the disease was not terminated by death, the destruction continued until the inflammation extended down through the epidermis (fig. 11). Invasion of the dermis first appeared around the lips of the epidermal cups at the hair follicles. This destruction may be the result of two forces operating simultaneously: first, pressure exerted by the ball of exudate within the cup; and second, the stretching of the epidermis at this point. The perifollicular area of the dermis was infiltrated with leukocytes, primarily neutrophils. Eosinophils were more numerous than in the earlier stages. The ulcers at times enlarged peripherally and united with neighboring ulcers. The inflammatory changes

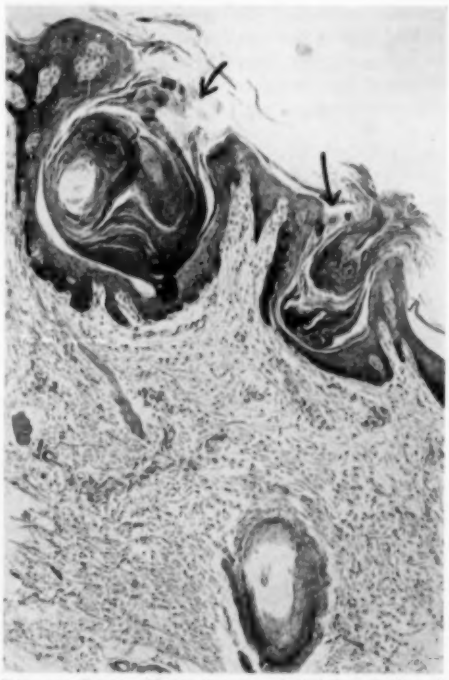

Fig. 7—Two epidermal cups containing balls of exudate (arrows) seen grossly as the brown spots described in the text. Abdominal area. x 90.

Fig 8—The early stages of the disease are indicated by dermal hyperemia and a slight amount of hyperplasia and hypertrophy of the stratum spinosum. Note the sweat glands (s). Inguinal area. x 90.

in the hair follicles sometimes proceeded below the level previously described and even extended into the base of the hair follicle, the sebaceous gland region, and the sweat gland region.

The severity of the inflammatory reactions varied with the individual. In general, the subacute form of the disease showed the same lesions as the acute form but, in addition, there was a greater

Fig. 9—The interpapillary pegs are lengthened and thickened. Capillary dilation and hyperemia are marked. Note the nucleated cells in the sebaceous gland (arrow). Observe the normal epidermal pigmentation. Axillary area. x 180.

Fig. 9a—Section of normal skin from axillary area of control pig. x 90.

amount of epidermal hyperplasia (fig. 12). The interpapillary pegs were longer, wider, and several displayed clubbing. Compound papillae were also formed in some cases.

The examination of skin sections, stained by the periodic acid-Schiff method, failed to reveal the presence of any recognizable specific causative agent.

Lymph Nodes.—The microscopic picture varied somewhat depending upon the se-

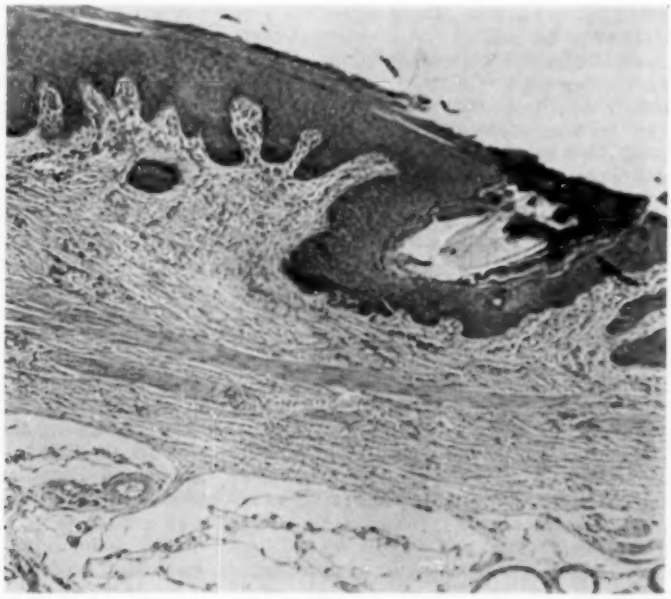

Fig. 10—A little later phase of the dermatitis shows increased hyperplasia and hypertrophy of the stratum spinosum with intercellular and intracellular edema. Nuclei are seen in the thickened stratum corneum. Inguinal area. x 90.

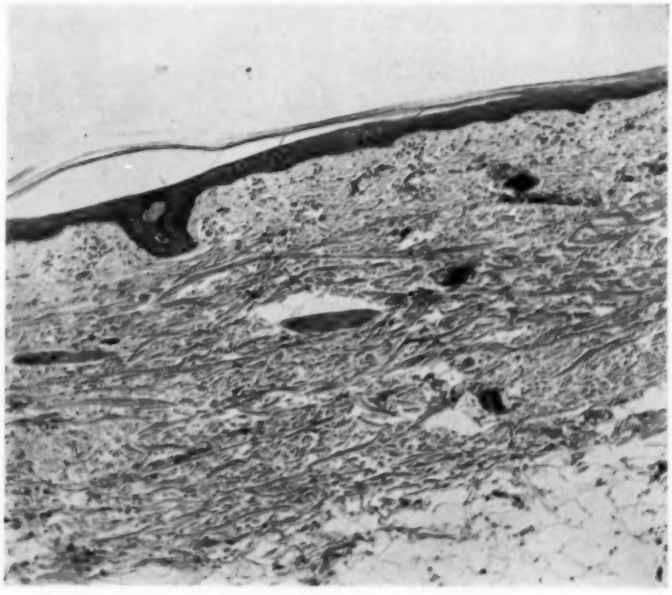

Fig. 10a — Normal skin from inguinal area of control pig. x 90.

verity of the dermatitis and the phase of the disease. There was always marked hyperemia and edema. At the same time there was hyperplasia of the reticular stroma and of lymphoid tissue. This often progressed to the degree that there was compression of both the subcapsular and peritrabecular sinuses. In addition, hyperplasia of the reticuloendothelial cells lining the sinuses was observed. Small areas of hemorrhage were commonly present. The free cellular elements included lymphocytes, free macrophages, a few monocytes, neutrophils, and plasma cells. Multinucleated giant cells were rarely encountered. The ever-present dense population of eosinophils was outstanding.

As the disease progressed, there were a few small groups of neutrophils in the cell-poor (medullary) area of the node. In a very few instances, these groups of neutrophils concentrated and enlarged into dense aggregates forming miliary abscesses. The typical picture, however, was that of serous lymphadenitis with eosinophilia.

Examination of the lymph node sections stained by the periodic acid-Schiff method, and by hematoxylin and eosin, failed to reveal any evidence of a recognizable causative agent.

Spleen.—No abnormalities were found.

Liver.—The microscopic findings were not very significant, usually hyperemia and cloudy swelling. In some livers, the sinusoids were normal and in others their lumen was narrowed. The interlobular septums were usually not very distinct, as would be expected in a young animal. Some of the livers showed mild fatty degeneration of the parenchyma. In one, necrosis was present in the periphery of some of the hepatic lobules. Except in this one instance, no inflammatory reaction was seen. No bacteria were found in any of the sections. In several livers, a few neutrophils were present. Eosinophils were found in all of the livers.

Kidney.—Cloudy swelling in varying degree was always present, especially in the convoluted tubules. In many instances, this process was advanced to cytoplasmic fragmentation and cellular disintegration. In a few specimens, there was mild fatty degeneration which appeared first in the tubules adjacent to the medullary rays.

Areas of hyperemia were present in a few specimens. There were several petechial hemorrhages in the papillary portion of the renal pyramids in one case.

Nearly all of the kidneys showed dilation of the collecting tubules. There was also moderate dilation of the glomerular spaces in several instances. In one there was a dilation of the collecting ducts and of the glomerular spaces, with resultant compression of the glomerular tufts.

Some homogeneous and granular precipitate, mixed with fragmented cells and a few erythrocytes, was seen in the collecting

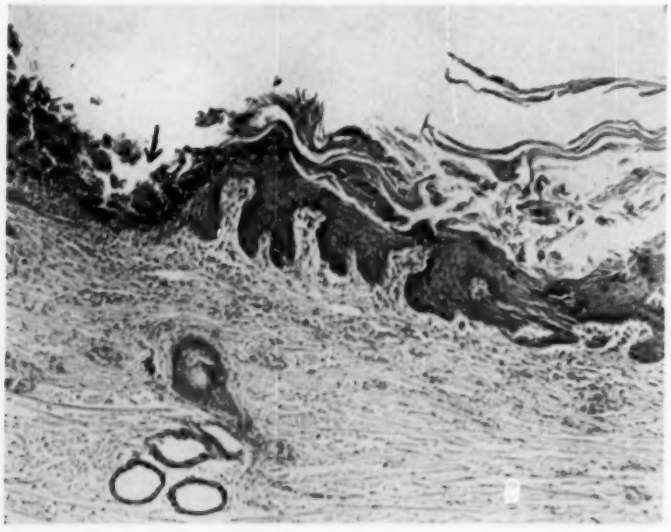

Fig. 11—Total epidermal destruction (arrow) resulting in the formation of a dermal ulcer. Note the leukocytic infiltration in the involved region. Inguinal area. x 90.

tubules and ducts of a few kidneys. (This material is apparently the precipitate reported in the gross findings. Hence, there is little doubt this condition existed in all the kidneys examined, only to be effaced by the processing procedures.)

Hyperplasia of the epithelium lining the pelvis was always present. This was also seen in the larger collecting ducts of a few kidneys. In addition, many of the pelvic epithelial cells showed hydropic degeneration, ballooning, and desquamation. Their cytoplasm was vacuolated, and contained some fine pale blue granular material. The other epithelial cells were compressed, contracted, and more deeply stained.

There was a small amount of material in the pelvis composed of precipitated proteins, fragmented cells, and erythrocytes.

An occasional leukocyte was observed in the renal parenchyma or contained in the blood vascular contents.

Bacteriological Findings.—Bacteriological studies on the blood and parenchymatous organs were negative but microorganisms were isolated from the lymph nodes from 2 pigs; one an acute, the other a subacute form of the disease. The organisms were identified as *Micrococcus epidermidis* (Winslow and Winslow) Hucker. They fulfilled the critera for this nonpathogenic organism as outlined in "Bergey's Manual of Determinative Bacteriology."[1]

Discussion

This investigation has been limited to the clinical and pathological aspects of a severe dermatitis of immature swine in Iowa. A complete explanation of all the features must await identification of the causative agent. Stokes[58] has proposed ten etiological factors for dermatitis. Three of these which might be concerned in this study are: the metabolic factor; the allergic or hypersensitivity factor, either general or specific; and the pyogenic factor (virus and bacteria). In addition, a fourth, the mycotic or fungous infection factor, was given consideration but has been eliminated by use of the periodic acid-Schiff staining methods.

Metabolic (or Nutritional) Factor.—Studies associating nutrition with the development of dermatitis are contained in reports by Hogan,[24] Chick *et al.*,[7] Unna,[53] Luecke *et al.*,[33] Witz and Beeson,[57] Hentges *et al.*,[22] Schreiner *et al.*,[55] Hansen *et al.*,[21] and Miller *et al.*[43] Also, it is generally accepted that nutritional diseases of mammals may be reflected through two, and sometimes three, generations.

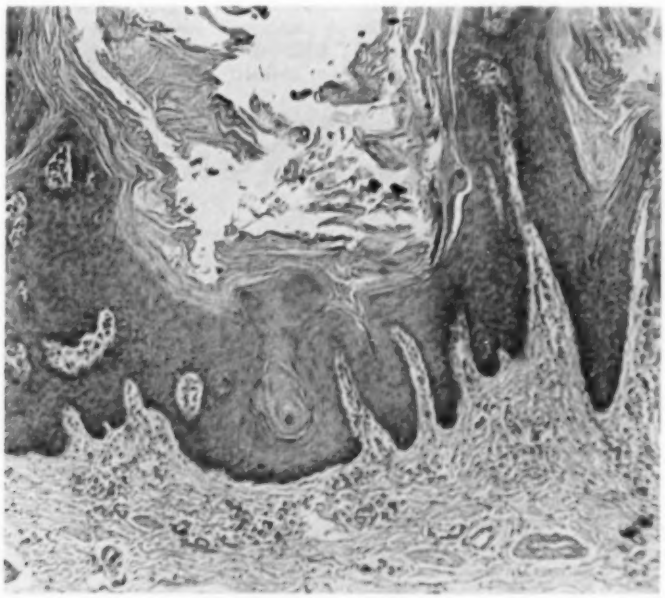

Fig. 12 — Photomicrographs of a skin section removed from the specimen shown in figure 6. Subacute form. x 90.

Allergy Factor.—The role of allergy in domestic animals is referred to by Brownlee [5] and Reddin.[50] The role of auto-antibodies in diseases of the skin is referred to in reports by Zoutendyk and Gear [68] and Marshall *et al.*[39] Weil and Reddin [64] produced allergy in cattle with extract of common ragweed and thus produced a heat-stabile neutralizing antibody and a heat-labile complement-fixing antibody.

A discussion of allergy can never be completed without directing attention to the eosinophil. This mysterious cell is discussed by Rebuck,[49] Cape,[6] and Best *et al.*[2] Eosinophilia is one of the many features of exudative epidermitis. It appears early and increases as the disease progresses.

Pyogenic Factor.—The histopathological picture of this condition reveals a bacterial flora found to be constantly present on the skin surface as a benign resident, noninvasive, but spreading as the tissues are destroyed. Obviously these organisms are saprophytic, not pathogenic. If they were truly pathogenic, there would be an equal inflammatory response. The histological picture did not demonstrate a specific causative agent.

Many features of this disease bear semblance to the diseases known to be caused by both the pox group and the foot-and-mouth disease group of dermatropic viruses. The possibility of a variant form of a virus should not be overlooked. Kelser [26] was convinced of the potential adaptation of viruses to different host species and different tissues.

Further studies of this disease might be enhanced by the use of capillary microscopic examinations of the skin as described by Gilje *et al.*[16]

SUMMARY

A clinical and pathological description is presented of an acute generalized dermatitis involving the entire body surface of pigs 5 to 35 days old. It is characterized by hyperhidrosis, excess sebaceous secretion, exfoliation, and exudation but no pruritus. It usually terminates in death. A secondary bacterial invasion accompanies the process.

This disease has been reported with increasing frequency and in some droves it has caused serious losses.

The material used in the study was taken from 20 droves, comprising 374 litters, and totalling 3,055 pigs. Over 40 necropsies were performed. Bacteriological and histopathological studies were made of lymph nodes, livers, kidneys, spleens, and skin.

Of the 3,055 pigs studied, the morbidity was 28.8 per cent and the mortality was 19.5 per cent. The mortality rate varied greatly.

No predisposing factors could be found. Peracute, acute, and subacute forms have been described.

Except for the skin lesions, no significant gross lesions were observed. *Micrococcus epidermidis,* a saprophyte, was isolated from the lymph nodes of 2 pigs but fungi could not be found.

Additional studies should be made to ascertain the cause and devise methods of control.

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Airborne Transmissible Gastroenteritis

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THERE have been reports of airborne chronic respiratory disease,[1] infectious bronchitis,[2] and Newcastle disease[2,3] of poultry. Transmissible gastroenteritis (TGE), a highly infectious disease of the intestinal tract of baby pigs, has been reported by Lee *et al.*[4] as also being spread by routes other than direct contact. This is a report on airborne transmissible gastroenteritis.

PROCEDURE

A room with a door in each end was divided by a wooden partition. Six modified Horsfall-Bauer[5] units were placed on each side of the partition. One side contained pigs which were intentionally infected and was designated side I. The other side, designated side C, contained control pigs which were exposed to the air from side I. Each unit on side I of the partition was placed so it could be connected to a unit on the other side (side C). The six units on side C were connected to an exhaust fan. The units on side I were open to the room while the units on side C were open only to the units on side I and to the exhaust fan. Therefore, the air moved from a unit on side I through a duct to a unit on side C and then to the exhaust fan. The length of the duct or the horizontal distance between the paired units I–1 and C–1, and I–2 and C–2 was 23 in. The distance between the paired units I–4 and C–4, I–5 and C–5, and I–6 and C–6 was 15 in. horizontally and 27 in. vertically for a total distance of 42 in.

Table 1 presents the air velocity for each pair of cages. The pigs on each side were fed and observed by separate personnel with separate cleaning rooms and facilities. Prior to each trial, all of the pipes and units were scrubbed with hot water and a detergent and then cleaned with hot water and steam. Feeding pans were scrubbed with a detergent and hot water after each feeding. The diet consisted of evaporated homogenized cow's milk which was reconstituted by mixing 2 parts of milk with 1 part of water. The pigs were offered milk three times each day.

Starting with the time at which the pigs on side I were fed the TGE infective material, trials 1, 2, and 3 were, respectively, of 120, 72, and 66 hours' duration. At the end of each trial, the pigs were necropsied.

Four litters, involving 23 Hampshire pigs, were used. They were obtained at 3 to 4 days of age. Litter mates of approximately equal weight were placed, 1 each, in the paired or connected Horsfall-Bauer units. Twenty-four hours after the pigs were placed in the units on side I each was fed about 2 ml. of a TGE suspension mixed in the milk. This suspension was prepared by mixing 100 Gm. of gastrointestinal tract taken from a TGE-infected pig with 1,000 ml. of tryptone broth. This mixture was centrifuged and the supernatant fluid used to infect the baby pigs. This TGE agent was recovered from an Illinois field outbreak four years ago and has been used for experimental transmission since that time.

Three pairs of pigs were successively subjected to the treatment in the connected pens I–1 and C–1; two pairs in pens I–2 and C–2; three pairs in I–4 and C–4; and two pairs in I–5 and C–5.

Three pigs were successively placed in pen C–6 and each time pen I–6 remained empty. This was done to check for any occurrence of spontaneous digestive disturbances which could have originated at the source of pig supply, or could have been due to contamination in the room air, or have been transferred by personnel at feeding time.

RESULTS AND DISCUSSION

Each pig intentionally infected on side I vomited or had diarrhea or both within 24 hours following the addition of the infective material to the milk. Thus an incubation period of 18 to 24 hours occurred

TABLE 1—The Number of Pigs and Their Average Weight for Each Unit, the Distance Between the Paired Cages, and the Air Velocity for the Paired Cages

Units	Distance between units, (in.)	Air velocity (cu. ft./ min.)	Av. body weight of pigs (Gm.)	No. of pigs with TGE symptoms
I–1	23	38	1527 (3)*	3
C–1			1556 (3)	3
I–2	23	15	1706 (2)	2
C–2			1746 (2)	2
I–4	42	53	1477 (3)	3
C–4			1546 (3)	3
I–5	42	16	1606 (2)	2
C–5			1853 (2)	2
I–6	42	....	..... (0)	....
C–6			1864 (3)	1

* Number in parentheses indicates the number of trials or pigs per unit.

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on side I. Within 18 to 24 hours after the appearance of vomiting or diarrhea by the intentionally infected pig on side I the paired pigs on the side C vomited or had diarrhea. Fewer pigs vomited on side C than on side I, but all had a diarrheal condition. The variations of distance and of air velocity in the experiment had no apparent influence upon the incubation period or the severity of the disease. These trials indicated that the air coming from the pens on side I at the time of the appearance of the initial vomitus or diarrhea was bearing infective material. Pigs on side C when exposed to this air developed typical symptoms of TGE. These trials did not establish the source of the airborne virus. It may have come from vomitus, feces, or exhaled air.

With each trial, unit 6 on side I was maintained empty and its respective unit on side C contained 1 pig. The air to the pig in unit C–6 came through leaks in the cage and pipe fittings from both sides I and C. This was arranged to evaluate the possibility of the pigs on side C being infected prior to the time they were put into the units, and to evaluate the possibility of infection occurring on side C from sources other than via the air duct connected to a cage containing an infected pig. In two trials, the pig maintained in this manner on side C did not show signs of TGE infection.

The pig (C–6) in the third trial was necropsied 90 hours after isolation or 66 hours after the pigs on side I were fed the infective material and at that time showed moderate diarrhea and mild gastritis. This pig possibly came in contact with the TGE agent at one of the times it was fed.

SUMMARY

Transmissible gastroenteritis (TGE) was airborne for distances of 23 and 42 in. at air velocities of 15 and 52 cubic feet per minute. The infectious material apparently became airborne at the time of the initial appearance of vomitus or diarrhea. Incubation periods for 4- to 6-day-old Hampshire pigs varied from 18 to 24 hours.

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Fescue Lameness in Cattle
I. Experimental Production of the Disease

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FESCUE lameness, a noninfectious disease of cattle, is characterized by dry gangrene of the hind feet and tail, and is caused by excessive consumption of tall fescue grass, *Festuca elatior* var. *arundinacea* (Schreb.) Celak. Cattle severely affected with the disease may die or recover with permanent defects of the feet. Animals mildly affected may recover completely following temporary lameness.

This study was conducted to determine (1) the ability of tall fescue grass to produce lameness, and (2) the type of lesions which characterize the disease. The specific toxic agent of tall fescue grass is being considered in an accompanying article by Maag and Tobiska.[6]

REVIEW OF LITERATURE

Cunningham[1] reported in 1949 that in New Zealand lameness developed in cattle which grazed continuously or for short periods on tall fescue grass. The incidence was higher and the disease more severe in cattle which grazed for short periods on pasture whose forage was predominantly tall fescue grass. The lesion was dry gangrene of one or both hind feet and extremity of the tail. The disease was produced experimentally in cattle by feeding dried immature tall fescue grass harvested prior to the formation of seeds. Three cattle were used experimentally. In 1 animal, lameness developed in a hind foot after 15 days of continuous feeding and in the other hind foot after 17 days of continuous feeding. The second animal became lame after feeding on the grass for 21 days when approximately 300 lb. of the grass had been consumed. The third animal did not develop lameness. In the opinion of the authors, ergot, not discernible in the grass, was not the cause.

Goodman[2] in 1952 reported the occurrence of fescue lameness in cattle of western Colorado where tall fescue grass grows in abundance. The disease developed during late fall and early winter months when cattle were forced to subsist on roughage which was predominantly tall fescue grass. One herd numbered 240 feeder cattle; of these, 65 became lame because of gangrene of one or both hind feet.

Stearns[3] in 1953 described lameness in cattle in Kentucky, which had grazed on cultivated grass, Kentucky fescue 31. In one herd of 40 Herefords, 8 cattle developed lameness of one or both hind feet with lesions of dry gangrene.

Beach[4] observed lameness of the hind feet with gangrene in cattle in Colorado which grazed on the grass, Kentucky fescue 31. Forty-five animals were grazed on the pasture during December, 1954, and January, 1955. Lameness developed in 15 cattle; gangrene formed in one hind foot of each of 2 animals.

Fig. 1—Laceration of skin proximal to the heel in a steer which grazed on tall fescue grass pasture. x 1.0.

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Knaus [5] encountered 69 cases of fescue lameness in cattle in the area of Rifle, Colo., during the period 1951 through 1955. The monthly incidence for these 69 cases was as follows: 8 cases in October, 25 in November, 5 in December, 14 in January, 11 in February, 4 in March, and 2 in May. No cases were seen in April, or in June to September, inclusive.

MATERIALS AND METHODS

Cattle.—Fourteen healthy animals of mixed breeds approximately 9 months of age were obtained for experimentation. Their physical condition was fair. Nine of these cattle were purchased and used experimentally at Rifle, Colo. Five were purchased and used experimentally at Fort Collins, Colo.

Pasture.—Approximately 10 acres of pasture were obtained at Rifle. The predominating grass was tall fescue. Small amounts of other grasses and clover were mixed with the tall fescue. The pasture was irrigated during the late dry summer.

Hay.—In October, 1954, much of the ungrazed fescue grass was moved, sun-cured, and baled. Some of the bales were used in the feeding experiment conducted at Rifle, Colo. The balance of the bales was transported to Fort Collins, Colo., for the feeding experiment. Mountain grass hay, which consisted of sedges (Carex spp.), rushes (Juncus spp.), and tufted hair grass (*Deschampsia caespitosa* [L.] Beauv.), was harvested in the valley of South Park, Colo., and was used to feed 3 cattle at Fort Collins.

Experiment 1.—Seven cattle which were purchased at Rifle remained in the pasture from August 7 through Dec. 27, 1954. Forage of the pasture was the only source of feed. The animals were observed daily for lameness.

Experiment 2.—Two cattle purchased at Rifle were confined to a feeding pen at Rifle from November 13 through Dec. 15, 1954. During this 32-day period, the animals were fed fescue grass hay exclusively and were observed daily for lameness.

Experiment 3.—At Fort Collins, 2 cattle which originated from Fort Collins were confined to a feedlot from Dec. 18, 1954, through Feb. 7, 1955, and were fed the tall fescue grass hay exclusively. Three similar cattle were confined to a separate pen from Dec. 28, 1954, through April 1, 1955, and were fed only the native mountain grass hay. All animals were observed daily for lameness. At necropsy all affected feet were dissected carefully.

TABLE 1—Lameness in Cattle Grazing Tall Fescue Grass Pasture

Animal No.	Entered pasture	Days to lameness
1	8-7-54	No lameness
2	8-7-54	50
3	8-7-54	No lameness
4	8-7-54	60
5	8-7-54	60
6	8-7-54	65
7	8-7-54	70

RESULTS

Experiment 1.—The cattle on pasture were unthrifty and consumed the tall fescue grass only when other more palatable species of forage were not available. After approximately six weeks in the pasture, linear lesions developed in the skin of the hind feet of most animals immediately proximal to the coronary bands of the heels (fig. 1). Presumably, these lesions originated as lacerations from the sharp serrated borders of the grass leaves. The lesions healed after several weeks. Table 1 presents the date of entrance to the pasture, interval to lameness, and specific animals which became lame.

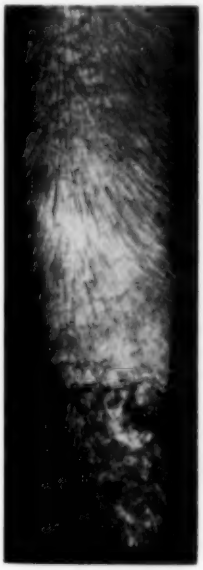

Fig. 2—Dry gangrene of extremity of the tail from a steer which grazed tall fescue grass for 70 days. x 1.2.

TABLE 2—Lameness in Cattle Fed Tall Fescue Grass Hay at Rifle, Colorado

Animal No.	Initiated feeding	Days to lameness
8	11-13-54	18
9	11-13-54	27

Experiment 2.—The 2 cattle, which were fed tall fescue grass hay at Rifle, showed lameness of the hind feet because of gangrene. Table 2 presents initial date of feeding and the interval to lameness.

Experiment 3.—In the experiment con-

Fig. 3—Axial surface of medial digit of left hind foot from a steer which grazed tall fescue grass for 70 days and developed gangrene of the left hind foot. The lateral digit has been removed. x 1.4.

a—Dorsal metatarsal artery; *b*—dorsal common digital artery; *c*—proper digital artery which is thrombosed; and *d*—bulbar arteries which are thrombosed.

Fig. 4—Dry gangrene of the right hind foot of a heifer which consumed tall fescue grass for 70 days. Separation of the gangrenous digits from viable tissue is developing. x 0.9.

Fig. 5—Dry gangrene of the lateral digit of the left hind foot of a steer which grazed tall fescue grass for 70 days. x 0.9.

a—Point of separation of gangrenous digit from viable tissue above.

ducted at Fort Collins, the 2 cattle which were fed tall fescue grass hay developed lameness of the hind feet because of gangrene of the extremities, while the 3 cattle which were fed mountain grass hay remained normal. Appetite of the cattle persisted throughout the experimental period. Table 3 presents date of initial feeding, interval to lameness, and total period of feeding.

PATHOLOGICAL CHANGES

Affected feet were gangrenous. Of the 14 experimental cattle, including 3 controls, 4 developed lameness in both hind feet. One developed lameness in one hind foot and in one front foot, and 3 developed lameness in one hind foot only. Two animals showed gangrene of the extremity of the tail (fig. 2). In affected feet, early stages showed necrosis and fissuring of the skin immediately proximal to the coronary band of the heel. Advanced stages showed dry gangrene of affected digits with a prominent line of demarcation at the junction of necrosed and viable tissues. Anatomically, the line of demarcation was located at any point distal to the

TABLE 3—Lameness in Cattle Fed Tall Fescue Grass Hay at Fort Collins, Colorado

Animal No.	Type of hay	Initial feeding	Days to lameness	Days on feed
10	Tall fescue	12-28-54	22	30
11	Tall fescue	12-28-54	42	50
12	Mtn. grass	12-28-54	No lameness	94
13	Mtn. grass	12-28-54	No lameness	94
14	Mtn. grass	12-28-54	No lameness	94

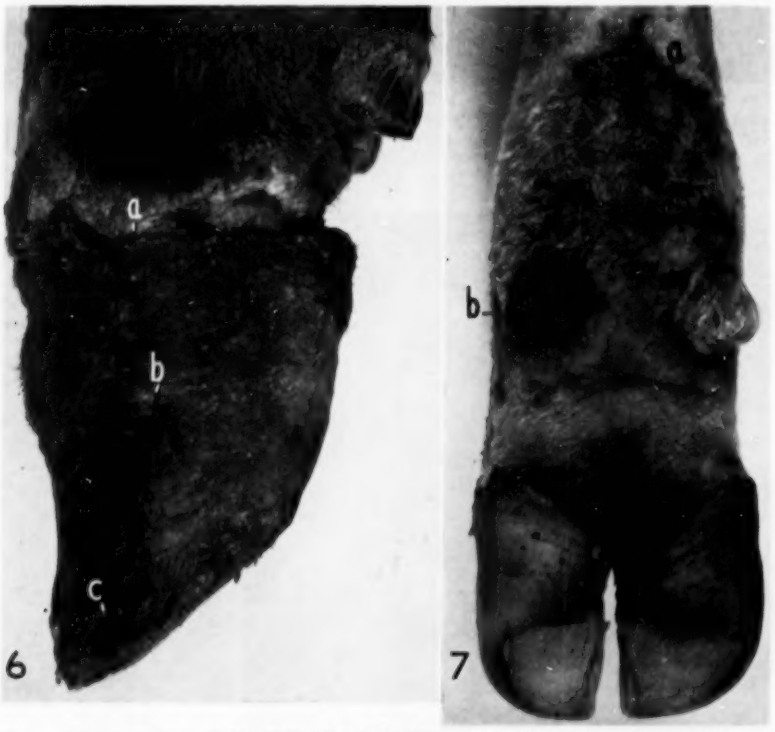

Fig. 6—Dry gangrene of the digit of a hind foot of a heifer which grazed tall fescue grass for 60 days. The necrosed hoof has detached and sloughed away. x 0.9.

a—Point of separation of gangrenous digit from viable tissue above; *b*—coronary band; and *c*—laminae.

Fig. 7—Dry gangrene of hind foot of a steer which consumed fescue grass hay for 27 days. x 0.5.

a—Point of demarcation between gangrenous tissues below and viable tissues above; and *b*—left dew claw from which the horn has detached.

Fig. 8—Dry gangrene of a hind foot of a heifer which grazed fescue grass for 60 days. The gangrenous digits have detached and left the distal extremity of the second phalanges exposed (a). x 0.7.

middle of the metatarsus. Usually, both digits of a foot were affected, although in some feet a single digit was gangrenous. Commonly the hoofs and hair detached from the dead tissue. Eventually the entire gangrenous part of the digits sloughed. Dissection of the vascular system showed thrombosis of the arteries in some affected feet (fig. 3). Figures 4 through 9 show gangrenous feet.

DISCUSSION

The concentrated incidence of the disease during months of late fall and winter suggests that low environmental temperatures contribute to the cause of the lesions. According to present interpretation, the toxic agent of the tall fescue grass causes vasoconstriction and predisposes to thrombosis. Ischemia plus low environmental temperatures cause gangrene of the extremities of the posterior limbs and occasionally the forelimbs and tail.

SUMMARY

Three experiments were conducted to determine the ability of tall fescue grass,

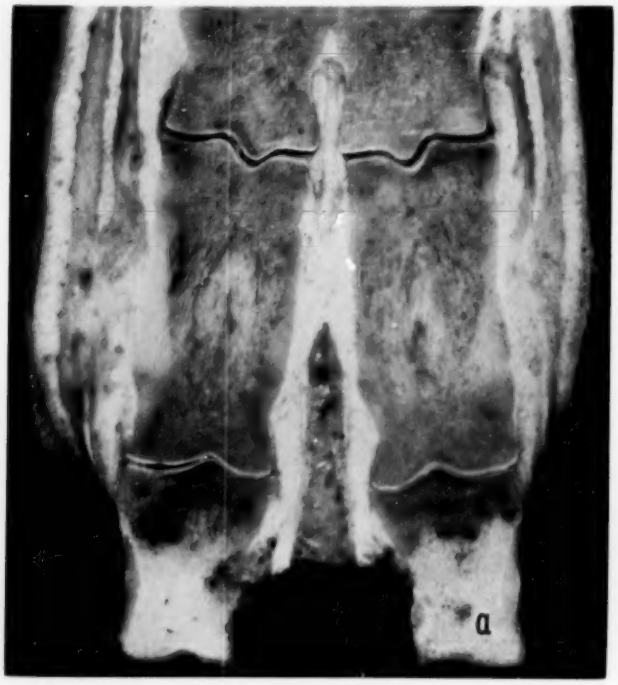

Fig. 9—Frontal section of same foot as in figure 8 showing the necrotic distal part of the second phalanx (a). x 0.9.

Festuca elatior var. *arundinacea*, to produce lameness in cattle. In experiment 1, 7 cattle were grazed on tall fescue grass pasture; 5 became lame. In two other experiments, 4 cattle were fed tall fescue grass hay exclusively; all became lame after 18 to 42 days. Three similar animals fed mountain grass hay which contained no fescue grass remained normal.

The lesions which were dry gangrene usually occurred in the distal extremities of the hind feet and, less often, in the extremities of the tail and front feet.

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Fescue Lameness in Cattle
II. Ergot Alkaloids in Tall Fescue Grass

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TALL fescue grass, *Festuca elatior*, var. *arundinacea* (Schreb.) Celak., grows in the vicinity of Rifle, Delta, and Montrose, Colo. For many years lameness, especially of the hind feet, developed in cattle which grazed this grass during late fall and winter months.

Lesions in affected cattle resembled lesions of cattle affected with ergot poisoning.

This study was an integral part of experimentation on fescue lameness. The lesions of affected cattle and experimental reproduction of the disease is reported in an accompanying article by Jensen, Deem, and Knaus.[9] The objective of this specific study was the identification of the toxic agent in the tall fescue grass.

REVIEW OF LITERATURE

In 1932, ergot was discovered in range grasses in Kansas.[1] Several cows on a farm in northeastern Kansas were grazing a cornstalk field and were also fed wild hay. Affected cattle became lame. The disease was diagnosed as ergotism after ergot was discovered in the wild hay and after the disease was found not to be contagious.

Another instance of acute ergot poisoning occurred in 170 Hereford calves, in California in 1954,[2] which were grazing 140 acres of irrigated pasture, consisting mostly of clover and native grasses with some wild rye. The calves developed lameness and extreme nervousness. However, no cases of the gangrenous form of ergot poisoning were observed. An examination of the grass revealed that 75 per cent of the rye grass was infected with ergot.

In certain areas of New Zealand, tall fescue grass forms a large part of the plant growth. Some cattle grazing these areas become affected with what is popularly known as "fescue foot."[3]

Ergot is a fungus *(Claviceps purpurea)* which develops on grains such as rye, wheat, and barley, and on many types of grasses such as fescue, blue grass, western wheat grass, red top, timothy, wild rye, brome, quack grass, and needle grass.[4]

Much research has been conducted to determine the active principles in ergot, and it has been established, mainly as a result of Barger[5] and his co-workers, that six pairs of alkaloids are usually present in ergot. In each pair of alkaloids, which are optical isomers, one possesses physiological activity and the other has little or no action. Besides alkaloids, other substances which are not alkaloidal in nature are present in ergot. Among the latter are a number of amino acids, simple nitrogenous bases, and a pigment, sclererythrin, which gives the fungus its characteristic purplish red color. The alkaloids in ergot on species of grasses and cereal grains vary in kinds and amounts. Also the alkaloids in ergot differ in their specific effects on the animal body. This accounts for the difference in symptoms of ergotism in different localities.[6]

MATERIALS AND METHODS

Grass.—Samples of the same grass that was used in experiment 3 of the accompanying article by Jensen *et al.*[9] were obtained. The tall fescue grass was from an experimental pasture near Rifle, Colo. Animals which consumed this type of grass became lame. The mountain grass hay, which consisted of sedges (Carex spp.), rushes (Juncus spp.), and tufted hair grass (*Deschampsia caespitosa* [L.] Beauv.) was harvested in South Park, Colo. Animals which consumed this type of grass remained normal.

A visual examination for sclerotia of ergot in samples of the above grasses was made.

Samples of the above grass were ground in a Wiley mill using a fine mesh screen.

Figures 1 and 2 show *Festuca elatior* var. *arundinacea* (Schreb.) Celak.

Chemical tests.—Qualitative tests for selenium,[7] sclererythrin of ergot, and ergot alkaloids were made on samples of the tall fescue grass and of the mountain grass hay.

One hundred-gram portions of the finely ground grass were placed in 1-liter Erlenmeyer flasks. Ether was added and mixed until it was about an inch over the surface of the contents. The mixtures were acidified with 10 per cent alcoholic tartaric acid. After standing for 24 hours with occasional shaking, the mixtures were filtered. The green-colored ether filtrates were extracted with 15-ml. portions of 10 per cent sodium carbonate. The aqueous alkaline solution of the tall fescue grass was orange to red colored at first and upon standing for several days turned to a rose to violet. The corresponding solution of the mountain grass hay was light yellow.

Several sclerotia of ergot of rye were treated in the same manner and the resulting aqueous alkaline extract was rose colored.

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Another 100-Gm. portion of each sample of ground grass was placed in 1-liter Erlenmeyer flasks. Ether was added as before and then the mixtures were made alkaline with 10 ml. of 1:10 NH_4OH. After standing for 24 hours with occasional shaking, the mixtures were filtered. The ether filtrates were acidified with 10 per cent alcoholic tartaric acid and then evaporated to dryness on a water bath. The residues were taken up with 50 ml. of water and made alkaline with 1:10 NH_4OH. These aqueous solutions were extracted with ether in portions of 40, 25, 20, and 15 ml. The ether extracts were combined and washed with three 25-ml. portions of water to which 1 ml. of 1:10 NH_4OH had been added. The ether extracts were then washed with two 25-ml. portions of water to remove the ammonia. The ether solutions were then extracted with 1 per cent tartaric acid in portions of 10, 10, and 5 ml. The tartaric acid extracts were evaporated on a water bath to 15 ml. to remove the ether.

Five milliliters of fluid extract of ergot were treated in the same manner to obtain a similar 1 per cent tartaric acid extract.

Two milliliters of p-dimethylaminobenzaldehyde reagent* were added to 2-ml. portions of each of the tartaric acid extracts. The extract from the fluid extract of ergot was deep blue. The extract from the sample of tall fescue grass was light blue and the extract from the mountain grass hay was colorless.

RESULTS

Visual.—No sclerotia of ergot were detected by a careful examination of the samples of grass. On some of the tall fescue grass a dark-colored substance was found at the nodes of the stems and axils of the leaves.

Chemical.—The chemical tests for selenium in the samples of grass were negative.

The rose color of the aqueous alkaline extract of the sclerotia of ergot of rye was due to the pigment, sclererythrin. The similar extract of the sample of tall fescue grass had a rose to violet color which indicated that some ergot pigment was present.

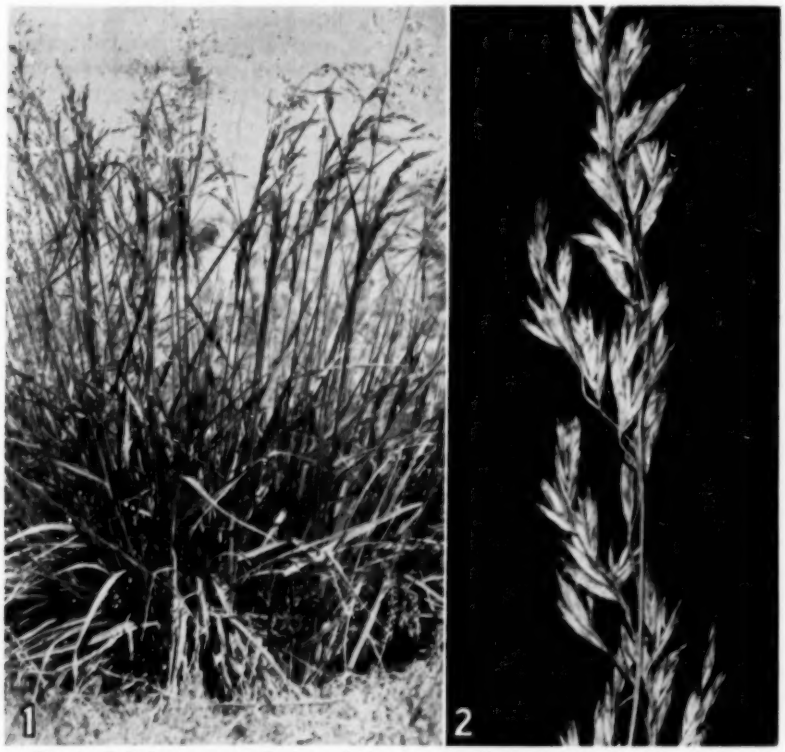

Fig. 1—Tall fescue grass, Festuca elatior var. arundinaceae, growing in a pasture. x 0.1.

Fig. 2—Seeds from mature Festuca elatior var. arundinaceae. x 1.5.

No ergot pigment was present in the mountain grass hay.

The deep blue color formed by adding the p-dimethylaminobenzaldehyde reagent to the final tartaric acid extract of the fluid extract of ergot was due to ergot alkaloids. The similar extract of the tall fescue grass was light blue which indicated that ergot alkaloids were present. No alkaloids were detected in the mountain grass hay.

TABLE 1—Spectrophotometric Analyses of Extracts from Grass and Sclerotia of Ergot of Rye

	Max. absorption (mμ)	Min. absorption (mμ)
Extract from sclerotia of ergot of rye	335; 465; 510	460; 492
Extract from tall fescue grass	334; 465; 505	460; 480
Extract from mountain grass hay	310;	None

1 per cent tartaric acid was used for the blank and also to dilute the samples.

TABLE 2—Spectrophotometric Analyses of Tartaric Acid Extracts of Grass and Fluid Extract of Ergot

	Max. absorption (mμ)	Min. absorption (mμ)
Extract from fluid extract of ergot	315	274
Extract from tall fescue grass	313; 315	272; 275
Extract from mountain grass hay	none	none

1 per cent tartaric acid was used for the blank and also to dilute the samples.

Spectrophotometric.—The final aqueous alkaline extracts of the samples of ground grass and the sclerotia of ergot of rye were neutralized with dilute HCl and spectrophotometric data obtained (table 1). Data were obtained also from the tartaric acid extracts (table 2).

Discussion

The chemical tests and spectrophotometric data indicate that some form of ergot was present in the tall fescue grass. No ergot sclerotia were detected by a visual examination of the tall fescue grass before it was ground in the Wiley mill. This seems to indicate that some form of ergot is capable of developing in the grass in locations other than in place of the seeds. The possibility exists that the plant tissue may produce a substance that may cause a toxic material to form.

Summary

Representative samples of tall fescue grass from an experimental pasture near Rifle, Colo., and mountain grass hay from South Park, Colo., were tested for ergot. The results of chemical and spectrophotometric analyses indicated that alkaloids, similar to those in ergot, were present in the tall fescue grass.

A qualitative test for selenium was made on samples of tall fescue grass and mountain grass hay and none was detected.

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Reports of Liver Glycogen Determinations by Biopsies on Dairy Cows

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WHILE studying ketosis at the University of Pennsylvania's New Bolton Center, it would have been most helpful to have known whether the liver of a cow with ketosis was depleted of glycogen, or whether the glycogen was present but the liver's mechanism of release was unable to function.

Liver biopsies have been conducted on farm animals by several investigators [1, 3] interested in determining some particular phase of liver function as a part of research projects.

Investigation of the literature reveals several basically similar techniques for conducting liver biopsies. All of these procedures resorted to a punch and aspiration technique. Bone [2] recently reported on an aspiration technique which used a modification of the Iversen-Roholm aspiration biopsy needle, and since the sample of liver tissue which he was able to obtain was of suitable weight for our investigation, this method was adopted.

MATERIALS AND METHODS

In the hope that a safe, practical, and accurate method of measurement of glycogen content in the livers could be developed, 7 normal (so far as ketosis was concerned) purebred Guernsey cows were assigned for study. Six of the 7 were lactating in fair condition, while cow A was badly emaciated and near death. She was destroyed after the first biopsy. Three of the cows had been bred and 4 had not.

Blood sugar determinations by the Folin-Wu method were taken prior to taking biopsies.

Liver Biopsy.— Arrangements were made for the manufacture of a proper instrument for making biopsies on livers of dairy cows.

The biopsy instrument consisting of a trochar, a clean-out rod, and a cannula fitted to a syringe by an adapter, is adequately described by Bone. However, since the original instrument was described, the manufacturer had lengthened the bevel on the slip adapter of the syringe and the cannula as they had been too short and the cannula frequently became separated with a loss of vacuum at the precise moment when it was needed. The technique for its use is here described.

The procedure is simple. The cow, confined in an ordinary stanchion, shows little or no distress. An area 2 inches square, between the right twelfth and thirteenth ribs, and 12 to 14 inches from the dorsal midline, is clipped and prepared for an aseptic operation. The area is anesthetized by infiltrating the skin, intercostal muscles, and peritoneum with 5 cc. of 2 per cent procaine hydrochloride. A 1-inch incision is made in the skin, after which the assembled biopsy punch is passed through the skin incision, intercostal musculature, and the peritoneum. No restraint is necessary except occasionally during the introduction of the local anesthetic.

After piercing the peritoneum, the trochar is withdrawn and a 50-cc. one-hand syringe with a Leur-Loc adapter is attached to the slip adapter which fits firmly into the cannula. The syringe must be in perfect working condition so that a constant suction will be maintained. The cannula is pushed inward, downward, and forward toward the opposite elbow until it is felt to contact the liver. Moderate pressure is maintained against the surface of the liver while 10 cc. of vacuum is created in the syringe. The vacuum is increased to 40 to 50 cc. while the cannula is being pushed inward into the liver, and rotated clockwise so that a core of liver tissue will be cut. The cutting of tissue can be felt as the cannula is being introduced.

The cannula is now quickly withdrawn, the syringe disengaged, and the punch passed through the cannula to expel the liver section. The skin incision is closed with two simple interrupted sutures of No. 1 surgical silk and a topical wound powder applied. Sutures may not be necessary, but they

TABLE 1—Blood Sugars of Cows in Glycogen-Determination Project

Cow	Prior to biopsy			
	1-26-55*	1-27-55	2-4-55	2-9-55
S	38**	43	43	43
L	37	33	42	42
RW	41	41	42	42
A	41	Destroyed		
MA	42	42	40	43
V	42	43	39	38
MY	41	42	42	42

Three cows had been bred as follows: S, 12-2-54; L, 11-6-54; RW, 12-10-54.

* All blood samples taken at 9:30 a.m.; ** milligrams of glycogen per 100 cc. of blood.

From New Bolton Center, University of Pennsylvania, Kennett Square.

The skill of technician Laura Bramble and the cooperation of bacteriologist Roland Woodward in handling the weights and balances is gratefully acknowledged.

TABLE 2 — Determination of Glycogen in the Liver (mg./Gm. of Liver) of Normal Cows

Cow	1-27-55	2-4-55*	2-9-55*
S	4.70	3.02	2.14
		3.50	3.59
L	4.44	3.2	3.83
		3.0	3.56
RW	Fat	3.77	3.79
			2.15
MA	4.12	3.5	1.86
		4.7	1.50
A	2.22	Destroyed	
V	3.84	3.8	8.44
		3.0	3.79
MY	3.70	1.43	1.04
		1.95	1.95

* Two biopsy samples taken on these dates.

seem to make healing more rapid with less scar formation.

Laboratory Procedure. — The liver tissue was placed in tubes of 30 per cent KOH solution and taken to the laboratory.

The method of Good, Kramer, and Somagi[5] was used with modifications. A pipette was used to place 2 cc. of 30 per cent KOH solution in a test tube. The tube was weighed, then the liver specimen was dropped directly into the KOH solution and the tube was again weighed. The tube was placed in a boiling water bath until the tissue was in solution. It was then cooled and chilled in an ice bath.

Methyl alcohol was added in a 1.1 to 1.2 volume and the tube was heated in a boiling water bath until the mixture started to boil. It was then cooled to room temperature, centrifuged, decanted, and drained well. Normal H_2SO_4, 2.5 cc., was added then it was autoclaved for 15 minutes at 15 lb. of pressure, cooled and neutralized with NaOH, and diluted with H_2O to 5 cc. A protein-free filtrate was made and tested for blood sugar as follows:

$$\frac{(.08901)\quad (\text{reading of Klett})}{\text{weight of sample}} = \frac{\text{mg. of glycogen/}}{\text{Gm. of liver}}$$

RESULTS

Blood sugar determination results, taken before and after biopsy, are shown (table 1) as milligrams per 100 cc. of blood.

The results of the glycogen determinations are shown (table 2) as milligrams of glycogen per gram of liver.

Only one biopsy sampling was made per cow on Jan. 27, 1955, but two samplings per cow were made on February 4 and on Feb. 9, 1955.

DISCUSSION

No evidence of shock resulting from hemorrhage was observed during this investigation, nor was there any marked distress or serious loss of milk production.

After completion of this phase of the project and five days after the last biopsy, 4 of the cows were sent to slaughter. In all but 1 cow the only change found was a variable-sized plate of fibrinous tissue covering the injured surface of the liver. Upon removal of this layer of fibrin, small white scars could be seen on the liver surface, the results of previous biopsies. The fourth cow, in addition to the fibrinous deposit, had a moderate-sized subcapsular hematocyst of the liver with localized peritonitis and adhesions and a slight trace of hemorrhage in the peritoneal cavity. None of the remaining experimental cattle showed evidence of peritonitis. Two of the cows are still producing milk.

With this meager standard for comparison, it is hoped that determinations of glycogen in liver of cows with ketosis may be secured from time to time in various stages of the disease, and thus help create a better understanding of this puzzling metabolic disturbance. Perhaps this report will spur others to contribute their thoughts and experiences along this line.

CONCLUSIONS

The rapidity of response to intravenous injections of hydrocortisone, as demonstrated by many cows suffering from ketosis, would seem to indicate that their livers contain sufficient glycogen for recovery if the mechanism of release could function.

We believe that biopsies on livers of cows with ketosis and determinations of their glycogen content will shed some valuable light on this question.

The method of securing liver samples with the instrument as made is comparatively safe. The cost of manufacture was $45.

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Effects of Dietary Trace Minerals, Excess Calcium, and Various Roughages on Weight Gains of Hereford Heifers

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THE ADDITION of trace minerals to rations of ruminants is recommended in some geographical areas of the United States depending upon the status of the soil in which roughages are grown. It is known that soils high in calcium carbonate may produce deficiency diseases in plants since boron, iron, manganese, and other elements are rendered unavailable to the plant.[11] In view of this and the emphasis placed on mineral feeding to livestock, experiments were begun at this station in 1950 to study the effects of dietary trace minerals and excess calcium on growth, blood, and histopathological changes of cattle.[17,18] Details of the hematology and histopathology will be presented in a subsequent publication.[19]

Beeson[1] mapped out the mineral-deficient soils in the United States. In the course of time with advances in nutrition and continual harvesting of crops without proper fertilization of the soil, more mineral-deficient soils may be induced thereby producing feeds which will require added trace minerals for proper growth of livestock.

Glendening *et al.*[8] analyzed cattle feeds grown in north central Kansas for various minerals. They found that typical beef cattle rations would not be deficient in copper, manganese, or cobalt. The iron status was difficult to evaluate since the iron requirement of livestock needs further investigation. Swenson *et al.*[16] have shown that young dairy calves raised in this area on calf starters and home-grown roughages have a low mean erythrocyte corpuscular volume of 35 to 37 $c\mu$ as compared with 50 to 60 for normal values. Supplementing the rations of these calves with dietary trace minerals (Fe, Cu, Co, Zn, and Mn) increased the size of the erythrocytes, indicating that the calves had a subclinical anemia caused by a trace mineral deficiency. Since the cell size is small, it would lead one to believe that iron and copper might be involved. Later, Jones *et al.*[9] demonstrated that the same dietary trace mineral supplementation to calves from birth to 6 months of age increased the growth rate as well as the values for hemoglobin, hematocrit, and erythrocytes.

Bentley and Klosterman[2] and Klosterman *et al.*[10] found that trace mineral supplements increased the weight gains of steers fed a poor quality hay. Later Bentley *et al.*[3] showed that cobalt was the first limiting trace mineral deficiency. Other trace mineral deficiencies were not excluded. Smith and Cox[16] were unable to demonstrate any beneficial effect on weight gains in steer calves fed trace minerals for 135 days. However, the lot fed trace minerals brought a higher market price.

Considerable data have been published regarding the Ca:P ratio in rations for livestock. The National Research Council[13] states that Ca:P ratios between 1:1 and 2:1 are satisfactory for all species if the phosphorus intake is adequate. duToit *et al.*[6] fed rations to two groups of calves with Ca:P ratio of 1.0:1.4 and 1.0:0.3. These rations contained adequate calcium and phosphorus. Growth of calves was not affected by the different Ca:P ratios during the one-year experimental period. Theiler *et al.*[20] reported that phosphorus deficiency invariably leads to rickets or osteomalacia. When adequate phosphorus was fed with a Ca:P ratio of 2.5:1.0, bone lesions did not occur. Otto[14] fed 2 calves weighing approximately 600 lb. each an average daily intake pf 43.3 Gm. of calcium and 3.03 Gm. of phosphorus. This ration provided a Ca:P ratio of 14.26:1.0 with an inadequate quantity of dietary phosphorus and an excess of calcium. Severe anorexia developed

TABLE 1—Trace Mineral Mixture Fed to Calves in Experiments 2 and 3

Salt	Mg. of salt/100 lb. body wt./day	Elemental mineral (mg.)
$FeSO_4 \cdot \times H_2O$ (85% purity)	640.00	200
$CuSO_4$	50.22	20
$MnSO_4$	61.49	20
$ZnSO_4$	27.45	10
$CoCl_2$	40.25	10
Total	819.41	260

TABLE 2—Average Weights of 2 Animals per Group in Experiment 1 Expressed in Pounds

Rations	Time on exper. (months) 0	6	(gain)	12	(gain)
1	302.5	497.5	195.0	605.0	302.5
2	370.0	565.0	195.0	635.0	265.0
3	315.0	441.5	126.5	540.0	225.0
4	370.0	508.0	138.0	625.0	255.0
5	350.0	500.0	150.0	620.0	270.0
6	345.0	506.0	161.0	600.0	255.0
Av.	342.0	503.0	161.0	604.0	262.0

From the Physiology Department, School of Veterinary Medicine, Kansas State College, Manhattan.

Contribution No. 145, Department of Veterinary Medicine, Kansas Agricultural Experiment Station, Manhattan.

TABLE 3—Average Weights of 3 Animals per Group in Experiment 2 Expressed in Pounds

Rations	Time on exper. (months) 0	6	(gain)	12	(gain)
1	313.3	627.3	314.0	861.3	548.0
2	291.7	555.0	263.3	786.7	495.0
3	305.0	551.7	246.7	795.7	490.7
4	366.7	601.7	235.0	847.7	481.0
Av.	319.2	583.9	264.7	822.9	503.7

TABLE 4—Average Weights of 3 Animals per Group in Experiment 3 Expressed in Pounds

Rations	Time on exper. (months) 0	6	(gain)	12	(gain)
1	478.0	727.0	249.0	1046.0	568.0
2	474.0	753.0	279.0	1045.0	571.0
3	493.0	732.0	239.0	1021.0	528.0
4	494.0	737.0	243.0	1019.0	525.0
Av.	485.0	737.0	252.0	1033.0	548.0

shortly and, later, stiffness and debilitation of the animals were pronounced. Phosphorus supplements had to be given to prolong the life of the animals during the experimental period which lasted 11 months.

In two experiments in 1950 and 1951 lasting 140 and 151 days, Dowe *et al.*[5,6] fed steer calves different levels of calcium in the form of ground limestone with Ca:P ratios of 4.5:1.0, 9.0:1.0, and 13.6:1.0. The ratio for the control group was 1.7:1.0. They found that the feed required per 100 lb. of gain increased and the rate of gain decreased as the calcium in the ration increased. However, it appears that not until the calcium was fed at high levels of 9.0:1.0, and especially at 13.6:1.0, did the differences in rate of gain become significant.

Under natural conditions, rations consisting of legume roughages may have Ca:P ratios of 5:1 or 6:1,[12] *e.g.*, alfalfa hay 6:1, ladino clover 4.5:1.0, and lespedeza hay 5.5:1.0. On the other hand concentrates contain more phosphorus than calcium, *e.g.*, the Ca:P ratio of corn is 0.07:1.0, wheat 0.12:1.0, oats 0.21:1.0, and linseed meal 0.47:1.0. When combinations of roughages and grains are fed, a more desirable ratio is obtained. By using suitable calcium and phosphorus supplements,[13] a deficiency or excess of either of the elements is seldom encountered.

MATERIALS AND METHODS

In each of a series of three experiments, over a period of three years, 12 Hereford heifer calves purchased locally from the same herd were used in each experiment. Two calves were assigned to each of 6 groups in experiment 1, and 3 calves to each of 4 groups in experiments 2 and 3. The calves in each experiment were fed as follows for one year:

Experiment 1

Group 1—Basal ration (prairie hay, linseed meal, and iodized salt)
Group 2—Basal ration + trace minerals
Group 3—Basal ration + excess calcium
Group 4—Basal ration + trace minerals + excess calcium
Group 5—Basal ration + excess calcium + alfalfa hay
Group 6—Basal ration + trace minerals + excess calcium + alfalfa hay

Experiment 2

Group 1—Basal ration (prairie hay, alfalfa hay, yellow corn, linseed meal, and iodized salt)
Group 2—Basal ration + trace minerals
Group 3—Basal ration + excess calcium
Group 4—Basal ration + trace minerals + excess calcium

Experiment 3

Group 1—Basal ration (wheat straw, yellow corn, linseed meal, iodized salt, ground limestone ($CaCO_3$), and a vitamin A concentrate)
Group 2—Basal ration + trace minerals
Group 3—Basal ration + alfalfa hay
Group 4—Basal ration + trace minerals + alfalfa hay

The basal ration at the beginning of experiment 1 consisted of 1 lb. of linseed meal per head daily, iodized salt, and prairie hay *ad libitum*. The prairie hay was of low quality since it had been exposed to abundant rainfall after cutting. Iodized salt was fed at the rate of 2.5 Gm. per 100 lb. of body weight per day to all groups in the three experiments. Water was available at all times.

TABLE 5—Average Weights of Animals With Added Trace Minerals (Fe, Cu, Co, Zn, and Mn) to the Ration

Exper.	Ration	No. of animals	Time on exper. (months) 0	6	(gain)	12	(gain)
1	2	2	370.0	565.0	195.0	635.0	265.0
	4	2	370.0	508.0	138.0	625.0	255.0
	6	2	345.0	506.0	161.0	600.0	255.0
2	2	3	291.3	555.0	263.3	786.7	495.0
	4	3	366.7	601.7	235.0	847.7	481.0
3	2	3	474.0	753.0	279.0	1045.0	571.0
	4	3	494.0	737.0	243.0	1019.0	525.0
Total		18	Av. 391.5	616.5	224.9	823.06	431.4

TABLE 6—Average Weights of Animals Without Added Trace Minerals to the Ration

Exper.	Ration	No. of animals	Time on exper. (months) 0	6	(gain)	12	(gain)
1	1	2	302.5	497.5	195.0	605.0	302.5
	3	2	315.0	441.5	126.5	540.0	225.0
	5	2	350.0	500.0	150.0	620.0	270.0
2	1	3	313.3	627.3	314.0	861.3	548.0
	3	3	305.0	551.7	246.7	795.7	490.7
3	1	3	478.0	727.0	249.0	1046.0	568.0
	3	3	493.0	732.0	239.0	1021.0	528.0
Total		18	Av. 372.3	599.5	227.1	816.7	444.3

Trace minerals were mixed with the concentrate of the rations and fed at the rate of 200 mg. of iron, 20 mg. of copper, 10 mg. of cobalt, 20 mg. of manganese, and 10 mg. of zinc per 100 lb. of body weight per day (table 1). The quantities of trace elements are expressed in elemental form. The iron, copper, manganese, and zinc were fed as sulfate salts whereas cobalt was fed as the chloride. A commercial trace mineral mixture similar to that in table 1 was employed in experiment 1 while an analytical grade of trace minerals was used in experiments 2 and 3.

In other trace mineral experiments with dairy calves,[21, 22, 23] 20 mg. of iron, 10 mg. of copper, 1 mg. of cobalt, and 10 mg. of Mn per pound of feed were used. These quantities were not optimal but produced no clinical manifestations. In the experiments herein reported and that of Jones *et al.*[9] a more desirable iron:copper ratio was fed and zinc was included at the same level as cobalt.

Excess calcium in the form of ground limestone was mixed with the concentrate in experiments 1 and 2 to provide a Ca:P ratio of 10:1. Alfalfa hay was fed to groups 5 and 6 of experiment 1 at the rate of 1.5 lb. per head daily.

The basal ration at the beginning of experiment 2 consisted of 2.0 lb. of alfalfa hay per head daily (amount fed throughout experiment), prairie hay *ad libitum*, 1.25 lb. of linseed meal, 4.0 lb. of ground yellow corn per head daily, and iodized salt.

The basal ration at the beginning of experiment 3 consisted of 2 lb. of linseed meal, 6 lb. of ground yellow corn per head daily, wheat straw *ad libitum*, and iodized salt. Ground limestone and a vitamin A concentrate were included in this basal ration to meet the calcium and vitamin A requirements.[13] Two pounds of alfalfa hay per head daily were fed to the calves in groups 3 and 4.

The three basal rations met the protein, total digestible nutrients, dry matter, calcium, phosphorus, and vitamin A requirements for the animals to gain ¾ to 1 lb. per head daily [12] in experiment 1, and 2 lb. per head daily [18] in experiments 2 and 3. Adjustments were made in the rations in the three experiments to meet the requirements due to growth during the first eight months of the experiments. Thereafter, the animals were continued on the same rations.

The calves in each experiment were kept inside a building for the entire experimental period of 12 months and were weighed at monthly intervals. Fecal samples were checked for internal parasites prior to the experiments and phenothiazine was given to the calves infected with *Haemonchus contortus* prior to and during the early part of the experiments.

Results and Discussion

Tables 2, 3, and 4 show average weights and weight gains of the calves on the three experiments. In comparing the weight gains of calves fed trace minerals in experiment 1 with groups 1, 3, and 5 very little difference is found. However, the calves fed trace minerals or alfalfa hay or both (groups 2, 4, 5, and 6) had a better coat and general appearance when compared with groups 1 and 3. Excess calcium inhibited the growth rate of calves (tables 7 and 8). On the other hand, the weight gains in groups 1 and 2 (table 2) compared with those of groups 3, 4, 5, and 6 were not significantly different when analyzed statistically due to the small number of animals in each group. The feeding of 1.5 lb. of alfalfa hay per head daily to calves in groups 5 and 6 did not offset the impaired growth rate caused by

TABLE 7—Average Weights of Animals With Excess Calcium in the Ration

Exper.	Ration	No. of animals	Time on exper. (months) 0	6	(gain)	12	(gain)
1	3	2	315.0	441.5	126.5	540.0	225.0
	4	2	370.0	508.0	138.0	625.0	255.0
2	3	3	305.0	551.7	246.7	795.7	490.7
	4	3	366.7	601.7	235.0	847.7	481.0
Total		10	Av. 338.5	535.9	197.4	726.02	387.5

TABLE 8—Average Weights of Animals Without Excess Calcium in the Ration

Exper.	Ration	No. of animals	Time on exper. (months) 0	6	(gain)	12	(gain)
1	1	2	302.5	497.5	195.0	605.0	302.5
	2	2	370.0	565.0	195.0	635.0	265.0
2	1	3	313.3	627.3	314.0	861.3	548.0
	2	3	291.7	555.0	263.3	786.7	495.0
Total		10	Av. 316.0	567.2	251.2	742.4	426.4

excess calcium, but the alfalfa hay improved the coat as much as trace minerals did.

The weight gains in experiment 2 were not affected by the dietary trace minerals (table 3). The calves in groups 1 and 3 had larger weight gains than those fed trace minerals, but the differences were not statistically significant. The coats and general appearances of the calves were the same in all groups. Apparently the 2 lb. of alfalfa hay fed daily to each animal in the experiment prevented the response obtained in experiment 1 with trace minerals.

In experiment 3 neither the trace minerals nor the alfalfa hay altered the growth of the animals significantly (table 4). The general appearances of the animals were the same in the four groups. The wheat straw proved to be a very satisfactory roughage for cattle when the protein, total digestible nutrients, calcium, phosphorus, and vitamin A requirements were met by proper supplementation.

After the three experiments were completed, the data were pooled, wherever feasible, to determine what effects trace minerals, excess calcium, and alfalfa hay had on the growth rate of the calves. This increased the number of animals receiving the same treatment and left a comparable number of animals as controls for statistical analysis (tables 5 to 10). Since there were equal numbers of animals in all groups and the number of animals is less than 20, the G-test was used in analyzing these data.[7]

In comparing the weight gains shown in table 5 with those in table 6, it is evident that trace minerals did not affect the weight gains. The average gain at 6 months was 224.9 lb. for the trace mineral groups as compared with 227.1 lb. for the untreated calves. At 12 months, the gains were 431.4 lb. for those fed trace minerals and 444.3 lb. for the calves not supplemented with trace minerals. Apparently, roughages grown in this geographical area contain enough trace minerals to meet the growth requirements of yearling beef cattle fed a balanced ration. This conforms to the findings of Glendening *et al.*[8] who determined the trace mineral content of feeds by chemical analysis. On the other hand, these feeds did not contain adequate trace minerals for proper development of the coat when a poor quality roughage was fed as in experiment 1. In addition, the hematological data[19] will show that the health and well being of the animals are improved by the addition of dietary trace minerals.

The feeding of excess calcium in the form of ground limestone definitely inhibited the growth rate of the calves. The weight gains at 6 months shown in table 7 are significantly different (5% level) from those shown in table 8. At 12 months these differences approached the 5 per cent level of significance. In the course of time, the calves seemed to make an adjustment in tolerating the large quantities of the calcium carbonate. During the last third of the experiment, some of the calves fed excess calcium were receiving as high as 1.1 lb. of ground limestone per head daily. An important requirement in feeding large quantities of calcium is to feed adequate

TABLE 9—Average Weights of Animals With Alfalfa Hay in the Ration

Exper.	Ration	No. of animals	Time on exper. (months) 0	6	(gain)	12	(gain)
1	5	2	350.0	500.0	150.0	620.0	270.0
	6	2	345.0	506.0	161.0	600.0	255.0
3	3	3	493.0	732.0	239.0	1021.0	528.0
	4	3	494.0	737.0	243.0	1019.0	525.0
Total		10	Av. 435.1	641.9	206.8	856.0	420.9

TABLE 10—Average Weights of Animals Without Alfalfa Hay in the Ration

Exper.	Ration	No. of animals	Time on exper. (months) 0	6	(gain)	12	(gain)
1	3	2	315.0	441.5	126.5	540.0	225.0
	4	2	370.0	508.0	138.0	625.0	255.0
3	1	3	478.0	727.0	249.0	1046.0	568.0
	2	3	474.0	753.0	279.0	1045.0	571.0
Total		10	Av. 422.6	633.9	211.3	860.3	437.7

phosphorus and vitamin D. This has been confirmed by other investigators.[6, 20] The phosphorus requirement was met in the three experiments. The sun-cured roughages supplied enough vitamin D to meet the requirements of the calves kept inside and away from sunshine.

The differences in the weight gains of calves fed alfalfa hay (table 9) and those not fed alfalfa hay (table 10) are of no significance statistically. In experiments 1 and 2, the alfalfa hay was beneficial in producing desirable coats on the animals. Alfalfa hay serves as a good source of protein as well as other nutrients for cattle. In these experiments 1.5 to 2.0 lb. of alfalfa hay per head daily proved to be a valuable supplement when low quality prairie hay was fed. Trace minerals improved the ration but little when alfalfa hay was included.

Summary and Conclusions

1) Three experiments have been conducted on 36 Hereford heifer calves to determine the value of dietary trace minerals, harmful effects of excess dietary calcium, and comparative values of different roughages as alfalfa hay, prairie hay, and wheat straw in promoting growth.

2) The addition of trace minerals to yearling beef cattle rations adequately supplemented had little effect on the growth rate. The trace minerals improved the coats and general appearances of the calves when a poor quality roughage as prairie hay was fed. The prairie hay had been exposed to inclement weather after cutting.

3) Excess calcium with the Ca:P ratio of 10:1 and the phosphorus requirement being met impaired the growth rate of the calves (significant at 5% level).

4) Alfalfa hay fed at the rate of 1.5 to 2.0 lb. per head daily did not stimulate the growth rate of the calves with rations consisting of low quality roughages but adequate in protein, total digestible nutrients, calcium, phosphorus, and vitamin A. Alfalfa hay, like trace minerals, improved the general appearances of the calves; however, trace minerals were of little value in this respect when alfalfa hay was included in the rations.

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The Utilization of Intravenously Administered Glucose, Invert Sugar, and Fructose in Cattle

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THE DEMONSTRATION by Cori,[1] in 1926, that insulin affected phosphorylation of glucose and that specific hexokinases for the phosphorylation of fructose and glucose existed in the liver and muscles led to investigations with fructose as a therapeutic agent in diabetes mellitus and other disturbances of carbohydrate metabolism.

Miller *et al.*[2] found that intravenous fructose injections in normal subjects produced a much greater rise in blood pyruvate and citric acid than did glucose injections. Similar results were obtained when fructose was administered to diabetic individuals. These studies indicated separate metabolic pathways for glucose and fructose and that fructose metabolism was not affected in diabetes. Wierzushowski[3] showed that the utilization of fructose was not dependent on insulin and Vestling *et al.*[4] demonstrated that fructose was more easily phosphorylated than glucose. Other investigators[5-7] have demonstrated less urinary loss from intravenously administered fructose and invert sugar than from glucose injections. Measuring arteriovenous differences in man following fructose injections, Van Itallie *et al.*[8] found that peripheral tissues assimilated fructose directly.

When normal dogs were compared with dogs made acidotic with ammonium chloride, Mackler *et al.*[9] found that the rate of disappearance of glucose from the blood was slower in the acidotic than in the normal animal indicating that acidosis inhibited cellular uptake of glucose or processes of phosphorylation or both. Using depancreatized dogs deprived of exogenous insulin, Whittlesey and Zubrod[10] demonstrated that the effects of fructose administration were similar to those seen with the infusion of glucose with exogenous insulin. One of the effects was a decrease in blood ketones. On the other hand, glucose alone accentuated blood ketone values. Thus, in the absence of insulin, fructose was shown to be ketolytic in depancreatized, ketotic dogs. These findings suggested a comparative evaluation of the utilization of fructose and invert sugar in cattle since ruminants differ markedly from monogastric animals in their carbohydrate metabolism. If these sugars have an advantage over glucose, their use would be indicated as valuable therapeutic agents in many disturbances of ruminants where therapy with glucose is now employed, such as ketosis of dairy cattle.

MATERIALS AND METHODS

Twelve yearling Hereford heifers, weighing an average of 822 lb. and being fed a balanced ration, were used in this investigation. Each animal, at intervals of two weeks, was injected intravenously with either glucose, invert sugar, or fructose in 50 per cent solutions at the rate of 0.5 Gm. per kilogram of body weight. The invert sugar was prepared by mixing equal quantities of glucose and fructose so that the resulting solution contained 25 per cent glucose and 25 per cent fructose. The injection site was the jugular vein. The time required for the injection was approximately five minutes.

Blood samples were obtained from the jugular vein of each animal prior to each injection and at two minutes, 30 minutes, one, two, four, and six hours postinjection. A fluoride-oxalate-thymol mixture was used as the blood anticoagulant.[11]

Urine samples were collected prior to and at one, two, four, and six hours following the infusion. The total quantity of urine voided in six hours was collected from 5 of the 12 animals after each infusion. Urination was induced by perineal stimulation.

The blood and urine samples were refrigerated immediately upon collection and protein-free filtrates were prepared within three hours. The blood glucose was determined according to the method of Nelson[12] while blood fructose was determined by the method described by Roe *et al.*[13] Duplicate sugar determinations using an M-14 Coleman spectrophotometer were made on each blood and urine sample.

RESULTS

The average preinjection blood glucose value for the 12 animals (36 blood samples) was 50.6 ± 11.5 mg./100 ml. of blood. Two minutes after the intravenous administration of the sugar solutions (table 1), the average blood sugar value was 414.8 mg. The differences in blood sugar values among the three treatments were not significant statistically. Thirty minutes after

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TABLE 1—Average Total Blood Sugar Values (Glucose plus Fructose) and Standard Deviations Expressed as Milligrams per 100 ml. of Blood after Separate Intravenous Infusions of Glucose, Invert Sugar, and Fructose in the Amount of 0.5 Gm. per Kilogram of Body Weight

Time after infusion	Intravenous infusion *			
	Glucose	Invert sugar	Fructose	Av.
2 min.	395.5 ± 128.0 (12)†	447.2 ± 90.8 (12)	401.7 ± 80.8 (12)	414.8 ± 101.8
30 min.	166.9 ± 43.3 (12)	144.0 ± 38.1 (12)	149.2 ± 31.2 (12)	153.4 ± 38.1
1 hr.	123.8 ± 44.4 (12)	95.2 ± 28.2 (12)	112.9 ± 40.6 (12)	110.6 ± 39.1
2 hr.	98.9 ± 30.7 (11)	55.0 ± 20.0 (12)	67.2 ± 25.2 (12)	73.7 ± 30.9
4 hr.	71.4 ± 21.5 (10)	48.7 ± 13.9 (12)	62.1 ± 29.4 (12)	60.7 ± 23.8
6 hr.	55.4 ± 7.3 (9)	47.6 ± 4.1 (10)	63.3 ± 19.8 (10)	55.4 ± 13.8

* The average preinjection blood glucose value for the 36 blood samples was 50.6 ± 11.5; † numbers in parentheses are the number of blood samples; P—0.001 between means of invert sugar and glucose at 2 hr.; P—0.01 between means of glucose and fructose at 2 hr. and between means of invert sugar and fructose at 6 hr.

the infusions, the blood sugar values had dropped to an average value of 153.4 mg. During this period, a pronounced diuresis was evident in all animals following administration of glucose or fructose but to a lesser extent following invert sugar. The total blood sugar values were not altered significantly at the 30-minute or the one-hour period by the kind of sugar administered.

Two hours after the sugars were given, an analysis of variance of the data showed that a highly significant difference (1% level) in the total blood sugar values existed. Following the infusion of invert sugar, the blood sugar values had returned to normal in two hours. Two hours after treatment, the animals having received glucose had the highest blood sugar values. On further analysis of the data, the average difference of 43.9 between the animals given glucose and invert sugar was found to be significant at the 0.1 per cent level. The average difference of 31.7 between those given glucose and fructose was significant at the 1 per cent level.

During the next two hours, the total blood sugar of the group, that had previously been given glucose, decreased 27.5 mg. as compared with smaller decreases for the groups given the other sugars which had either reached or approached normal values two hours after infusion. Thus four hours after the infusions, no statistical difference was present among the mean differences of the three groups.

Six hours after treatment with glucose the total blood sugar values of the group had reached normal values. After fructose treatment, the group continued to have total blood sugar values slightly above normal. On analysis of the data, the six-hour values showed a significant difference among treatment means. The difference of 15.7 between the means following the fructose and invert sugar treatments was found to be significant at the 1 per cent level indicating that total blood sugar had not returned to normal in six hours following fructose infusion.

The urinary excretion data from 5 of the 12 calves given intravenous infusions of either glucose, invert sugar, or fructose are shown (table 2). The mean differences in the grams of glucose excreted in six hours were significant at the 1 per cent

TABLE 2—Average Urinary Sugar Excretions and Standard Deviations of 5 Cattle, Following Intravenous Infusions of Glucose, Invert Sugar, and Fructose

Total injection and excretions	Intravenous infusion		
	Glucose	Invert sugar	Fructose
Grams sugar injected *	194.6 ± 19.9	204.8 ± 14.3	197.2 ± 23.2
Grams glucose excreted in 6 hr.	20.8 ± 4.7	8.6 ± 4.77	4.3 ± 1.0
Grams fructose excreted in 6 hr.	0	6.1 ± 3.2	12.5 ± 4.4
Grams total sugar excreted in 6 hr.	20.8 ± 4.7	14.7 ± 6.2	16.8 ± 4.8
Percentage of sugar excreted in 6 hr.	10.9 ± 3.8	7.3 ± 3.5	8.5 ± 2.0
Milliliters of urine excreted in 6 hr.	4805 ± 1409	3211 ± 1272	4599 ± 1047

* 0.5 Gm. per kilogram of body weight.

level. The same is true for the mean differences in the amount of fructose excreted in six hours. With regard to the grams of total sugar (glucose and fructose) excreted in the urine, the differences among the three treatments were not significant; however, there was a trend indicating that less sugar was lost in the urine of animals treated with invert sugar. The difference in quantity excreted after treatments with invert sugar and fructose (2.1 Gm.) was less than between those given glucose and invert sugar (6.1 Gm.) and between glucose and fructose (4.0 Gm.).

The total quantity of urine excreted six hours after treatment (table 2) reveals that diuresis was less marked after the animals were given invert sugar as mentioned previously. When given glucose or fructose, the animals excreted comparable quantities of urine. The difference in the three tests approached the 5 per cent level of significance.

DISCUSSION

Working with Ayrshire, Red Poll, and Shorthorn adult cattle, Holmes [14] found that the blood sugar values returned to normal in one and one-half to two and one-half hours following the infusion of glucose at the rate of 0.3 Gm. per kilogram of body weight. He reported a 10.3 per cent sugar loss in the urine under these conditions. Bell *et al.*[15] likewise reported normal blood sugar values at two hours following glucose infusion. These workers reported an average renal glucose threshold of 98 mg./100 ml. of blood in cattle.

Baker *et al.*[16] in studying lipogenesis in the normal and alloxan diabetic rat and using C^{14} labeled acetate, lactate, and glucose have shown that the formation of fats from acetate and lactate was greatly depressed in the alloxan-treated animals. The capacity of the livers in such animals to convert glucose to fatty acids was impaired markedly. In their study, it was shown further that fructose feeding restored the ability of the diabetic liver to synthesize fatty acids from acetate and lactate in amounts similar to that synthesized when the diabetic rats were fed glucose plus insulin. This indicated that an active glycolytic system was necessary for lipogenesis. These findings pose the question whether fructose can correct the faulty carbohydrate and fat metabolism and the accumulation of ketone bodies found in ketosis of dairy cattle. Lehninger [17] in a review of fat metabolism stated that coenzyme A (CO-A) is essential in the oxidation and synthesis of fatty acids. Apparently the reactions in these processes are reversible with the same enzymes involved. Coenzyme A is likewise essential in the intermediary metabolism of carbohydrates and amino acids thus indicating a complex relationship in the metabolism of fats, carbohydrates, and proteins. This leads to the interesting possibility that fructose or invert sugar infused into a bovine animal with ketosis may restore the ability to utilize and metabolize fats by the normal pathways as well as provide an active glycolytic system necessary for fat metabolism to proceed normally.

The preliminary findings on normal cattle, reported herein, demonstrated that the blood sugar levels returned to normal more rapidly following the infusion of invert sugar. In addition, there was less sugar loss in the urine when fructose or invert sugar was employed than when glucose injections were used. This indicates that either fructose or invert sugar is more rapidly utilized, by hepatic and extra hepatic tissues, than glucose. This suggests a possible advantage of fructose or invert sugar in the treatment of cattle with ketosis.

SUMMARY AND CONCLUSIONS

1) A comparative study of the utilization of intravenous infusions of glucose, invert sugar, and fructose in cattle at the rate of 0.5 Gm. per kilogram body weight is reported.

2) Total blood sugar values returned to normal two hours following invert sugar treatment, six hours after glucose, and were slightly above normal six hours after treatment with fructose.

3) Marked diuresis occurred in animals following glucose and fructose infusions, with less diuresis after treatment with invert sugar.

4) There was a tendency for less sugar to be excreted in the urine following fructose or invert sugar than following glucose infusions. The fructose-treated and invert sugar-treated animals excreted similar quantities.

5) Thus the utilization of invert sugar by the body tissues over a given period of time was greater than that for either fructose or glucose alone.

6) The possible advantages of invert sugar therapy over glucose therapy in metabolic disorders of cattle such as ketosis are discussed.

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The Course and Pathogenicity of Initial Infections with *Dictyocaulus Viviparus*, the Lungworm of Cattle

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IN JULY, 1953, the writers began a series of tests to ascertain the course and pathogenic effects of lungworm infections produced by the administration of various dosages of infective *Dictyocaulus viviparus* larvae to calves and yearlings which had not previously been exposed to infection with this parasite. The results obtained are reported here.

When the tests were begun, the precise role of lungworms as a cause of fatal respiratory disease in cattle did not appear to be satisfactorily elucidated either by the experimental or field investigations that had been reported.

The results of only one experimental investigation on the effects of *D. viviparus* infections had been fully described;[9] none of the animals involved in it died, although up to 7,500 infective larvae were administered to some of them. Although deaths among experimentally infected animals had been briefly mentioned in two other reports,[5, 8] neither the numbers of larvae administered nor any other details were given.

Several investigators,[1-7, 10] mainly in Britain, of epizootics of bovine bronchitis and pneumonia had reported that adult cattle as well as calves and yearlings often are severely or fatally affected by *D. viviparus* infections. Some of these reports indicated that the importance of lungworms as a cause of bovine respiratory disease had been underestimated previously. However, still later British reports suggested that under epizootic conditions some fatalities may have been erroneously attributed to lungworm infections because another concurrent respiratory disease had been overlooked.

The probable identity of "fog fever" and atypical parasitic bronchitis was first reported[5, 7] in 1951 and 1952 on the basis of field studies. The experimental production of the fog fever syndrome in calves infected, or exposed to reinfection, with *D. viviparus* was briefly reported[8] early in 1953. Thus, these reports strongly suggested that a respiratory disease originally supposed to be of other origin may have been, in reality, a manifestation of lungworm infection. The implication that some deaths previously charged to lungworms may not have been due to them, or not entirely due to them, arose in 1953 when a new bovine respiratory disease, called "cuffing pneumonia," was discovered[11] in Scotland. It was reported to be associated with lungworm infections, but not caused by them. Some additional pertinent experimental data published by others after the present investigation was begun are mentioned in one of the following sections of this paper.

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MATERIALS AND METHODS

Of the 23 cattle used in this experiment, 21 were less than 2 weeks of age when purchased, and the remaining 2 were yearlings. Except for the latter 2, which had a few gastrointestinal helminths, all were worm-free prior to their experimental infection with lungworms.

The first 3 test calves received larvae cultured from feces from an experimentally infected "source" calf. The feces of these and of other test calves were the source of all larvae used later in the experiment.

Infective larvae for administration to test animals were obtained as follows: First-stage larvae were collected from the donor's feces by Porter and Cauthen's[9] method, except that Baermann screens were used instead of gauze. The larvae and any associated fecal material which settled through the screens were concentrated by centrifugation, washed several times with distilled water, and reconcentrated. This larvae-feces combination was mixed with charcoal in 4-inch Petri dishes. Enough additional distilled water was introduced to moisten the culture thoroughly. To maintain the moist atmosphere, the Petri dishes were covered and placed over water in a larger covered container. Infective-stage larvae developed in these cultures within seven days at room temperature. At this time, or a few days later, they were washed out of the charcoal-feces medium and concentrated by sedimentation and, if necessary, by centrifugation. The number of viable ones administered was estimated by a dilution-counting method. All active or tightly coiled ones in the samples, and some that were loosely coiled, or nearly straight, and quiescent at the moment of examination, were enumerated as viable. Only those quiescent ones were counted, whose appearance agreed with that of other individuals that had been examined for a longer time and seen to move, although they likewise were inactive when first observed.

The larvae usually were administered on the day of collection, but sometimes they were stored in water in a refrigerator for a few days before use, since this resulted in no appreciable loss of

TABLE 1—Data on Fatal Initial Experimental Dictyocaulus Viviparus Infections in 8 Calves and a Yearling

Animal No. and breed	Status when initially infected: Approx. age (months)	Status when initially infected: Weight (lb.)	No. of larvae administered	Interval from infection to death (days)	Patent period (days after infection)	Interval from infection to onset of symptoms (days)	Weight at death (lb.)	Lungworms at necropsy: No. recovered	Lungworms at necropsy: Developmental status (estimation of distribution): Mature	Lungworms at necropsy: Developmental status (estimation of distribution): Immature: Large	Lungworms at necropsy: Developmental status (estimation of distribution): Immature: Small	Size range: Males (cm.)	Size range: Females (cm.)
69X	2.0	145	10,000 S	28	†	14	145	550	++	+++	+	0.32–2.22	0.16–2.54
66G	2.5	137	15,000 D*	65	23–65**	26	128	450	+++	++	+	.....	1.0 –5.8
77H	2.0	150	30,000 S	22	†	12	105	6,100	–	+++	+	.....	.....
81H	2.0	167	40,000 S	22	†	11	137	6,350	–	+++	+	0.44–0.95	0.37–2.13
196X	4.0	184	50,000 S	23	21–23**	14	162	4,800	+	+++	+	0.47–1.95	0.30–2.34
95H‡	6.0	244	54,000 S	26	†	8	207	10,300	++	+++	+	.....	.....
186X‡	14.0	516	54,000 S	47	25–47**	18	426	2,400	+++	+	–	.....	.....
30H‡	6.0	350	205,000 S	26	†	9	308	12,300	++	+++	+	0.20–4.13	0.16–3.18
36H‡	2.0	120	750,000 S	7½	†	6	.....	18,200	–	–	+++	0.07–0.17	0.11–0.22

H—Holstein; X—Sindhi-Jersey; G—Guernsey; S—single dose; D—divided doses; * *see* table 3 for number and size of doses; ** larvae still present at death; † died in prepatent period; ‡ used as control prior to infection.

vigor. Administration was *per os* by means of a metal pipette attached to a rubber bulb.

Experimental animals were quartered individually in clean, well-ventilated concrete stalls. They were weaned at 6 to 8 weeks of age and then fed alfalfa hay and a grain mixture.

Starting before the end of the shortest reported prepatent period, i.e., the interval from infection to the first appearance of first-stage larvae in the feces, approximately 1-lb. fecal samples from each calf were examined by means of a Baermann apparatus, usually daily. After the first detection of larvae in such a sample at least once-weekly examinations of representative 100-Gm. samples were made and the number of larvae recovered determined by a dilution-counting method. When the number per gram became small, pound samples were again examined until larvae were no longer recovered. To estimate the total larvae output for a patent period, all feces were recovered from the pen floor as often as possible and the 24-hour output weighed. The per-gram counts for days intervening between the twice weekly, or more frequent, counts were estimated.

Routine clinical examination of infected animals was made from three to five times a week during the critical stages of infection and less frequently thereafter. These examinations included observation of the pulse, respiration, and temperature, with special attention to auscultation of the lungs.

Differential blood counts, hematocrit determinations and weights were usually obtained at weekly intervals; during noncritical periods of the infections, the interval was occasionally two, or rarely, three weeks.

Necropsies were performed in a routine manner except for examination of the lungs. The bronchi and bronchioles were thoroughly opened and all macroscopic worms removed for fixation. The lungs were then incised, placed in large containers of water and squeezed by hand. The lung was removed after at least 6 hours in the water and liberated worms were recovered by sedimentation and preserved in formalin.

A dilution-counting method was used to estimate the total number of worms recovered. If none were macroscopic, the sedimented material was suspended and the microscopic worms in each of a series of samples were counted. If mature and other sizeable worms were present, they were cut into small segments which were added to the sedimented material. Suspensions were then effected and the tail segments and any microscopic worms in each sample were counted. In certain cases, measurements to determine the size range of the worms were made.

Typical pathological areas of the lungs and regional lymph nodes were removed and fixed in 7 per cent formalin for later sectioning. Lung tissue and regional lymph nodes from all but one of the fatal cases were submitted for bacteriological examination to the Bacteriology and Mycology Section, Animal Disease and Parasite Research

Branch, Agricultural Research Service, Beltsville, Md.

Results and Observations

Survival and Larval Dosage.—Nine of the 22 test animals died of pneumonia or other lung involvements, caused directly or indirectly by their lungworm infections (table 1) and 13 survived (table 2). Since the survivors were kept for tests on acquired resistance (to be reported), direct evidence as to the worm loads they initially acquired is lacking. However, their infections apparently were rather heavy in some cases, as indicated by the numbers of larvae found in their feces (table 2). The damage caused by these infections can be estimated only clinically.

All of 7 animals, 6 young calves and a yearling, given a single dose of 30,000 or more larvae died. However, 2 animals, a calf and a short yearling, given about 43,000 larvae in divided doses survived. Apparently, death or survival were determined more by the number of larvae in the initial dose than by the total number given.

Although all of 3 calves given 5,000 and all of 3 given 20,000 larvae survived, 1 of 2 given 15,000, and 1 of 5 given 10,000 larvae died. Thus survival was the rule if 20,000 or fewer larvae were given either in a single dose or in divided doses.

Susceptibility to Infection.—All animals listed in tables 1 and 2 were susceptible to infection on initial exposure. Moreover, either fecal or necropsy examinations, or both, showed that mature worms developed in 19 of the 22. There was no evidence of inability of the worms to reach maturity in the other 3 calves (table 1). One of them (36) died only seven days after infection. The remaining two (77, 81) lived beyond the minimal time which the worms require to reach maturity. Some of the worms recovered from them at necropsy were nearly mature, and these infections undoubtedly would have matured had the hosts lived a few days longer.*

Prepatent and Patent Periods.—Sixteen of the animals survived long enough for determination of the prepatent period of their infections; its length varied from 21 to 30 (average 25) days. The patent period was usually short, varying from 27 to 72 (average 41) days, in 12 of the 13 animals which survived their infections. There was no clear relation between length of the prepatent or patent period and the age of the host, the number of larvae administered, or the administration of single versus divided doses.

Peak and Total Larval Outputs.—The peak number of larvae per gram (l.p.g.) observed in the feces of 11 of the animals which survived their infections varied from 7 to 642 (table 2); for calf 66 and yearling 187, which died three weeks or more after their infections became patent (table 1), these values were 162 and 650 l.p.g., respectively. The peak outputs tended to be highest in infections in the animals that received 43,000 to 54,000 larvae and lowest in infections produced by 5,000 or 10,000 larvae. The two highest

* Mature infections developed in 2 of 3 yearling, or older, cattle, not included in this experiment because of their obscure history regarding prior exposure to lungworms.

TABLE 2—Data on Nonfatal Initial Experimental Dictyocaulus Viviparus Infections in 12 Calves and a Yearling

Animal No. and breed	Status when initially infected: Approx. age (months)	Status when initially infected: Weight (lb.)	No. of larvae administered	Prepatent period (days)	Maximum No. of larvae per 1gm. of feces	Patent period (days)
83H	1.0	148	5,000 S	24	.....	41
85H	1.0	153	5,000 S	24	.....	43
93X	1.0	125	5,000 D*	26	42	52
61H	1.5	160	10,000 S	23	34	30
68X	2.0	125	10,000 D*	25	277	72
82H	4.0	243	10,000 S	28	7	32
97H	2.0	144	10,000 S	28	227	45
86H	0.5	92	15,000 S	25	547	....
91H	3.0	145	20,000 D*	24	207	34
96H	2.0	150	20,000 S	28	481	31
99A	1.0	.....	20,000 S	30	118	27
187X	11.5	450	43,000 D*	25	642	37
193X	4.5	263	43,000 D*	25	90	47

H—Holstein; X—Sindhi-Jersey; A—Angus; S—single dose; D—divided doses; * *see* table 3 for number and size of doses.

peak counts were in the feces of the oldest animals.

Estimated total larval outputs for five infections in calves and one in a yearling were: 2,700,000 (No. 93), 7,100,000 (No. 91), 9,350,000 (No. 97), 13,000,000 (No. 68), 15,000,000 (No. 96), and 33,000,000 (yearling No. 187). In a case where death occurred while the infection was still patent (yearling 186), the 23-day estimation was 27,000,000. The greatest outputs were in the feces of the oldest and most heavily exposed of these animals. Daily outputs exceeding 1,000,000 larvae were estimated for calf 96 on six days, yearling 186 on six days, and yearling 187 on 12 days; the output for No. 187 was in excess of 5,000,000 on one of these days.

TABLE 3—Divided Doses of Larvae Administered to 6 Animals Listed in Tables 1 and 2

Animal No.	Total No. of doses	Size of doses	Interval between successive doses (days)	Total interval between first and last doses (days)
93	4	700		
			2	
		900		21
			10	
		1,200		
			9	
		2,200		
68	10	1,000	Usually 1	12
66	30	500	Usually 1	40
91	10	2,000	Usually 1	13
187; 193	4	8,400		
			1	
		8,100		8
			1	
		13,200		
			6	
		13,300		

Contrary to normal expectations, the limited available data indicated that the total output was not necessarily less if the same number of larvae was given in divided doses versus a single dose.

Significance of Bacteriological Findings.—Bacteriological examination** of the lung and regional lymph nodes to detect the more common pneumonia-causing organisms was made in 8 of the 9 fatal cases (table 1). The only significant finding was the presence of *Pasteurella multocida* in the lungs and lymph nodes of calves 69 and 66 and yearling 186. The behavior of these animals suggests that this organism may have been an important factor in their death. For example, 8 other calves (table 2) survived the effects of a larval dose equal to, or greater than, the 10,000 given to calf 69. Also, calf 66 succumbed after receiving 15,000 larvae in divided doses, whereas calf 86 survived this number given in a single dose. Usually the same number of larvae had a lesser effect if given in divided doses (table 4). Moreover, 6 of the 9 deaths occurred in the fourth week of infection, and both of the calves given about 50,000 larvae died at that time. However, yearling 186, also given about 50,000 larvae, lived for three weeks beyond this obviously critical period suggesting that this older animal might have been able to survive the effects of its lungworm infection *per se*. Although the finding of Pasteurella in both of the calves which died at low dosage levels is considered significant in accounting for their exceptional behavior, it is not contended that fatal uncomplicated lungworm disease can not result from such low exposures to infection.

Worm Loads in Fatal Cases.—About 18,200 worms were recovered from the lungs of calf 36 about seven days after infection (table 1); all were immature and microscopic (length, 0.07–0.22 cm.). The worm counts in the other four cases in which pathogenic bacteria were not detected, and in the one not examined for bacteria, ranged from 4,800 to 12,300, whereas 450, 550, and 2,400 worms, respectively, were recovered in the three cases where secondary bacteria capable of causing pneumonia were detected. Although many of the worms recovered from the lungs of the 6 animals that died 22 to 28

TABLE 4—Effect of Administration of Larvae in Single versus Divided Doses on Rapidity of Onset and Severity of Symptoms

Animal No.	Total No. of larvae administered	Interval from infection to onset of symptoms (days)	Degree of reaction
83, 85	5,000 S	12	Severe
93	5,000 D*	41	Slight
61, 82	10,000 S	19, 22	Moderate
97	10,000 S	14	Severe
69	10,000 S	14	Fatal
68	10,000 D*	25	Moderately severe
96, 99	20,000 S	7, 16	Severe
91	20,000 D*	25	Slight
81	40,000 S	11	Fatal
187, 193	43,000 D*	22, 20	Severe

S—single dose; D—divided doses; *see* table 3 for number and size of doses.

** The writers are indebted to Drs. C. A. Manthi and E. R. Goode, Animal Disease and Parasite Research Branch, ARS, Beltsville, Md., for this phase of the investigation.

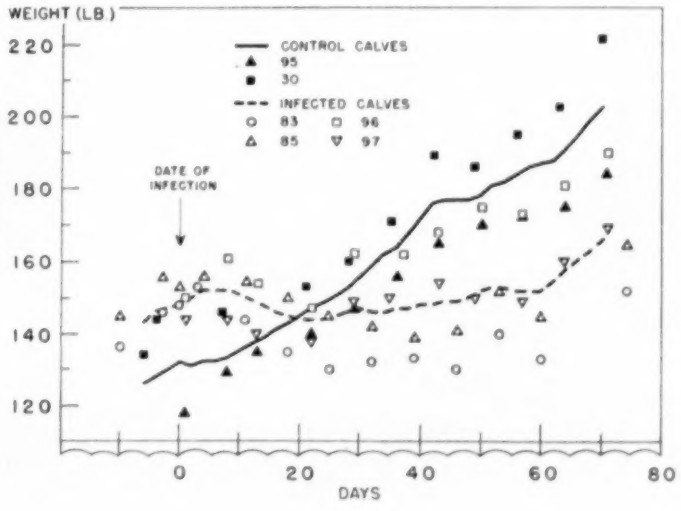
days after infection were mature, some small worms also were present. In four such cases where measurements were made, the smallest ones varied from 0.16 to 0.37 cm. in length. This wide range in size and degree of maturity after a single exposure to infection suggests a host resistance, either inherent or provoked by the first larvae to reach the lungs.

Larval Dosage and Signs.—In general, neither the rapidity of the onset nor the severity of the signs and effects of the infections was proportionate to the number of larvae administered. However, among the animals that received a single administration of larvae, there was some tendency for more prompt onset of signs with increased larval dosages. Usually animals given a particular number of larvae at one time reacted more quickly and more severely (table 4) than those given a comparable number in divided doses.

Respiratory Signs.—As early as seven days after exposure, and usually by the twelfth day, the respiration became more rapid, more forced, and more abdominal than normal. Sometimes the transition from normal to abnormal respiration was abrupt, occurring within a few hours. These early signs were accompanied, or shortly followed, by a cough which became more frequent and more intense as the disease progressed. Occasionally, violent "fits" of coughing occurred, resulting in one instance in an apparent temporary loss of consciousness. Abnormal lung sounds such as moist râles, emphysema, and wheezing were heard but did not necessarily reflect the clinical condition of the animals. For example, the pathological lung sounds were more distinct and more extensive in surviving calf 68, than were those up to the time of death in certain fatal cases.

In fatal cases, the initial respiratory disturbance became progressively more pronounced, some animals breathing with the mouth open, tongue protruding, and head lowered. A few of the animals occasionally exhibited a type of respiration that can best be described as resembling a dog panting. As respiration became progressively more difficult and the animal more distressed, coughing became softer and less frequent. The animal approaching death seldom coughed. A loud expiratory grunt developed in all cases that terminated fatally, as well as in some that survived. The survivors had an expiratory grunt for varying lengths of time but eventually lost it. It was audible from outside the barn in some instances. An accumulation of frothy material around the mouth was not uncommon; it was blood-tinged for two to three days prior to death in 1 animal.

Anorexia and Fecal Consistency.—In lightly infected animals, the appetite and water intake generally were most markedly affected when a diarrhea developed, as it did in cases 83, 85, 66, and 99. These diarrheic animals showed complete inappetence



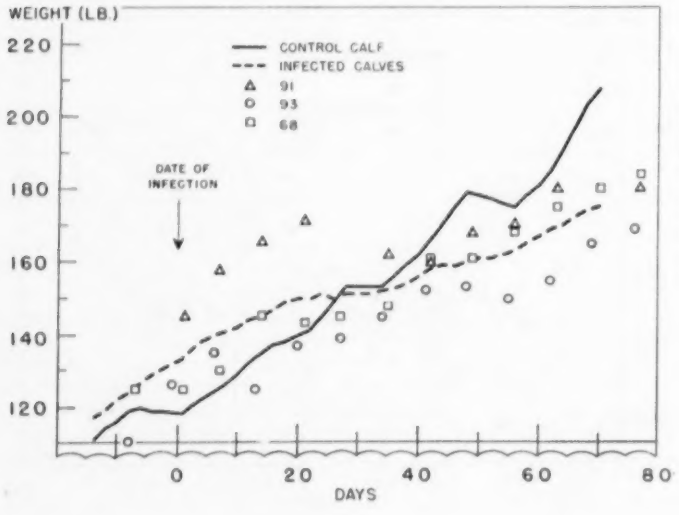
Graph 1—Comparison between average weight gain of calves 83, 85, 97, and 96, respectively given 5,000, 5,000, 10,000, and 20,000 infective larvae in a single dose, and of control calves 30 and 95 for 70 days following infection.

for varying lengths of time. In the other lightly infected and the moderately infected animals, the appetite and water intake were somewhat reduced. In heavily infected animals, appetite and thirst remained near normal until the disease had progressed so far that any attempt to eat or drink apparently caused considerable discomfort. As a result, these animals refused food and water, became gaunt and severely dehydrated, and their feces became abnormally dry; exceptions were calf 77 which had diarrhea for a week preceding death and calf 36 which died seven and one half days after infection and appeared normal in all respects up to the last 12 to 18 hours.

Elevation in Temperature.—A consistent, early temperature rise was not observed in these infected animals, nor was the temperature elevated in every case. The average peak temperature for the 13 animals that survived was 104 F. and the average interval from infection to occurrence of the peak was 33 days. The highest peak was 106.4 F. on day of infection 22 (calf 99); the lowest peak was 102.8 F. on day 21 (calf 61). The corresponding averages for the 7 animals that died during the prepatent period of their infections, or shortly thereafter, were 104.6 F. on day 16. In the two remaining fatal cases, the maximum temperatures were only 101.4 F. and 103.6 F. on days 13 and 57, respectively. These data indicate that the temperature response is not a reliable clinical sign in lungworm infections.

Effect on Weight.—A marked weight loss (up to 90 lb.) preceded death in seven of the eight fatal cases; 1 animal remained the same. Animals with sublethal infections did not gain as well as comparable controls, nor as well as in their preinfection periods, where such weights were available for comparison. The weight changes during sublethal, single dose infections in 4 test calves are compared with those of 2 control calves (graph 1). In ten weeks, the controls gained an average of 53 lb. (0.75 lb./day) more than the infected calves. Similar data are shown for 3 calves given comparable numbers of larvae in divided doses and for their control (graph 2). In ten weeks, the control calf gained 47 lb. (0.67 lb./day) more than the infected calves. A marked temporary weight loss (graph 3) was shown by yearling 187, which received 43,000 larvae in divided doses and by the end of ten weeks the control (yearling 186) had gained 88 lb. (1.26 lb./day) more than the infected yearling.

Effect on Blood.—Comparison of the hematocrit values of the blood of the 22 test animals showed no significant reduction in red cell volume percentage from their preinfection norms. Total white cell counts were not made. There was an apparently significant rise in eosinophile percentage in most of 18 animals for which



Graph 2—Comparison between average weight gain of calves 93, 68, and 91, respectively given 5,000, 10,000, and 20,000 infective larvae in divided doses (table 3) and of control calf 67 for 70 days following infection.

these data were obtained. In some instances, a maximum of 30 per cent was reached. The available data are not susceptible to detailed interpretation, but they suggest that more frequent observations might reveal a pattern of eosinophile response to initial *D. viviparus* infection.

Gross Pathology.—Beyond evidence of emaciation and dehydration, the significant lesions were confined to the lungs and regional lymph nodes. The only exception to this was in calf 69 which, in addition, showed a gastroenteritis and diffuse peritonitis.

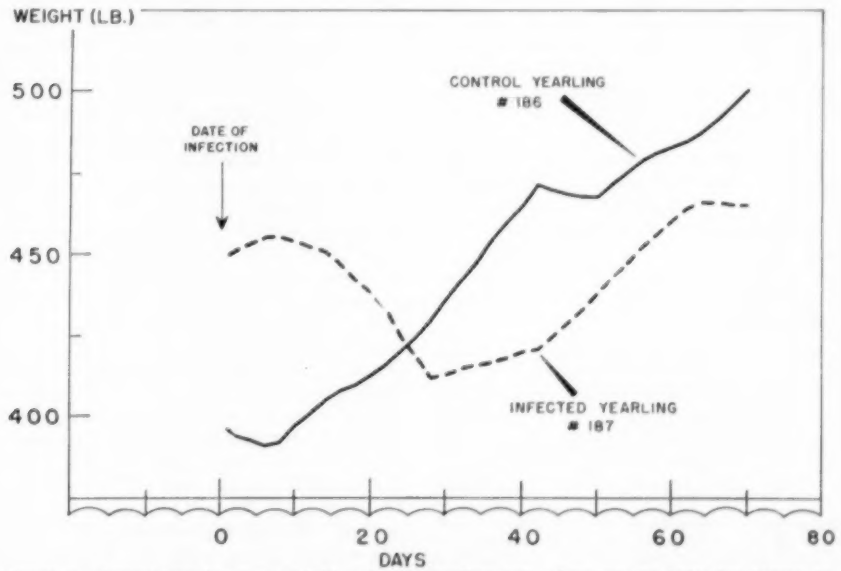
The gross appearance of the lungs resembled that seen in so-called shipping fever pneumonia. The degree of involvement varied from lungs showing only a few scattered pneumonic lobules to those showing pneumonic changes beneath practically the entire pleural surface.

Interstitial and subpleural pulmonary emphysema were constantly present, were usually widely distributed, and were most often found in the areas of least pneumonic change. In varying degrees, subpleural petechiae and ecchymoses were usually present. The regional lymph nodes usually were edematous or hyperemic or both but occasionally were normal in appearance.

Except in calf 36, which had only microscopic worms, the opened respiratory tree revealed varying numbers of lungworms distributed throughout but concentrated in the primary and secondary bronchi. In some, especially calves 95 and 30, the worms were so numerous in the secondary bronchi as to suggest a mechanical occlusion sufficient to cause death by asphyxiation. In addition to worms, an excessive accumulation of frothy fluid was in the air passages and was very suggestive of pulmonary edema. In two cases, the fluid was mixed with a thick green to grey mucopurulent material. The mucous membrane of the trachea, bronchi, and bronchioles was inflammed in calves 30 and 69.

The presence of macroscopic worms with some or all of the above mentioned lesions enables one to make a postmortem diagnosis of verminous pneumonia. However, animals comparable to calf 36 in which only microscopic worms were present, would offer more difficulty; not only were the worms invisible, but the infection was so acute that typical pneumonic lesions were not extensive enough to suggest pneumonia as the cause of death.

Histopathology.—The histopathological



Graph 3—Comparison between weight gain of yearling 187, given 43,000 infective larvae in divided doses (table 3), and of control yearling 186 for 70 days following infection.

findings here reported† are based on a thorough study of the lung tissues of fatal cases 30, 77, 186, and 196. Since these findings were uniform, similar studies of the other fatal cases are not included. The following is a composite description of the observed lesions:

Areas of consolidation involved portions of or entire lobules. The alveoli contained neutrophiles, mononuclear phagocytes, lymphocytes, Langhans-type giant cells and, occasionally, many eosinophiles; the latter were usually focal in concentration. Some alveoli contained hemorrhage; others, serous or fibrinocellular exudates. Round worm larvae, occasionally surrounded by eosinophiles, were encountered in the alveoli. Only rarely, areas of alveolar epithelization were observed. Arteries, veins, and alveolar capillaries were often distended with erythrocytes.

The bronchioles and secondary bronchi frequently contained a cellular exudate consisting of exfoliated epithelial cells, degenerating leukocytes, as well as normal neutrophiles, lymphocytes, mononuclear phagocytes, and round worm segments. The bronchiolar epithelium occasionally was multilayered and thickened. In yearling 186, peculiar papillary projections of the mucous membrane almost occluding the lumina of some of the bronchi and bronchioles and a mild lymphoid hyperplasia adjacent to some of the larger bronchi were observed.

Emphysema, both subpleural and interstitial, was common and widespread in all cases, the interstitial often being evidenced by blood vessels appearing free from the surrounding connective tissue. The emphysema was accompanied by areas of atelectasis, and interstitial hemorrhage was occasionally seen.

The over-all picture was one of verminous broncho-pneumonia with a densely cellular exudate.

DISCUSSION

The present results agree with Porter and Cauthen [9] in showing that sublethal experimental lungworm infections can cause severe respiratory disturbances and have a marked adverse effect on weight gains. They also confirm the above brief reports,[5,8] and the more detailed ones [12,13,15] which appeared after this work was begun, by showing that fatal lungworm disease can readily be produced experimentally. Michel [13] and Jarrett *et al.*[12] found that death rather often occurred about three weeks after infection. In the present series, 6 of the 9 fatalities occurred at about that time. However, no evidence was obtained to support Michel's [13] finding that a severe clinical response occurs in calves about the time they lose their infections.

In the present tests, only 2 of 13 animals that received from 5,000 to 20,000 infective larvae died and *Pasteurella multocida* was isolated from the lungs of both. Michel [13,15] found a dosage as low as 5,000 larvae to be fatal in many cases and many of the animals given 5,000 or 10,000 larvae by Jarrett *et al.*[12] died. However, some animals given as many as 50,000 larvae by Michel [15] survived.

Had the test animals been under more adverse environmental conditions and greater exposure to bacterial and viral respiratory disease the percentage of fatalities probably would have increased. Most animals in these tests survived exposure to as many as 20,000 larvae but, apparently as a result of the damage caused by such exposure, *P. multocida* may, as in calf 69, become a serious pathogen and contribute to, or cause, death. Also, prolonged, more or less daily, infection with a relatively small number of larvae, as in calf 66, and as presumably may occur on pastures, apparently can devitalize the lung or reduce resistance enough to allow *P. multocida* at least to contribute to a fatal pneumonia.

The etiological agent of the cuffing pneumonia described by Jarrett *et al.*[11,12] in some lungworm cases is uncertain, but may be viral. In the present work, no attempt was made to isolate any virus from fatal cases. However, none of the many other calves simultaneously housed in the same premises with the test animals suffered any respiratory disturbances. The histopathological findings in fatal cases did not show any peribronchiolar cuffing, described by Jarrett *et al.*[11] as a characteristic lesion of cuffing pneumonia, nor any extensive epithelization of the alveoli, such as they reported. Therefore, provisionally at least the writers must attribute death to lungworm disease *per se* in those cases where the bacterial findings were negative.

† The writers are indebted to the Armed Forces Institute of Pathology, Washington, D. C., for assistance in evaluation of the histopathology, and one of us (RR) is participating with them in the preparation of a detailed report on the gross and microscopic pathology of experimental lungworm infections.

Porter and Cauthen apparently concluded from their experiment that yearlings may be age-resistant to lungworm infestation to a considerable degree. In the present tests, yearling 186 was highly susceptible to infestation. Its death was not, however, definitely attributed to lungworm disease *per se*, for the reasons explained elsewhere. It also appears reasonable to think that such an animal, because of the larger size of its lungs, might tolerate the same degree of exposure to, and infection by, lungworms that appeared to be fatal *per se* to young animals.

Attention has been called to the presence of microscopic worms in the lungs of calves three to four weeks after initial infection. This phenomenon, which is interpreted as evidence of resistance, also has been reported by Michel.[15] Death caused solely by microscopic forms of *D. filaria* has been experimentally produced in sheep[14] and, in the present tests, a calf given a very large number of *D. viviparus* larvae died before the worms reaching its lungs had time to become macroscopic. In such a case, the cause of death can not be diagnosed by ordinary methods of examination of the lungs at necropsy. Workers investigating the etiology of pneumonic diseases in cattle in the field should be alert to the possibility of what might be termed "fatal larval lungworm disease" in young, as well as older, stock and should not exclude it as a factor except on the basis of suitable laboratory procedures.

It was estimated that 1 animal voided up to 33,000,000 first-stage larvae during the course of its initial infection. However, the inefficiency of the Baermann apparatus in the recovery of larvae is well known, and the actual total output probably was considerably larger than this figure indicates. The great extent to which an infected individual animal can quickly contaminate a pasture is further suggested by recovery of over 5,000,000 larvae from a 24-hour fecal output of 1 test animal. The great potential danger of such an individual to other stock is obvious since ingestion of a comparatively small number of infective larvae can result in the death of a susceptible calf.

SUMMARY

Twenty calves and 2 yearlings were given 5,000 to 750,000 infective larvae in single or divided doses and all were susceptible to initial infection with *Dictyocaulus viviparus*. Mature or nearly mature worms developed in all animals that lived three weeks or longer. The prepatent period varied from 21 to 30 days and the patent period from 27 to 72 days. All of 7 animals that received 30,000, or more, larvae in one dose died. Two of 13 that received fewer than 30,000 died, but *Pasteurella multocida* was considered a possible factor in their death. Coughing, râles, polypnea, pulmonary emphysema, expiratory grunt, pyrexia, dehydration, anorexia, diarrhea, and constipation were observed in the infected animals. However, neither the rapidity of onset nor the severity of the clinical signs was proportionate to the number of larvae administered. Sublethal infections caused poor weight gains. Significant worm loads were found in all of the nine fatal cases, but none of the worms in the lungs of a calf that died a week after it received 750,000 larvae were macroscopically visible. The presence of some microscopic immature worms three to four weeks after infection with a single dose of larvae indicated either a natural, or a rapidly acquired, resistance. Interstitial and subpleural emphysema were constant findings at necropsy, but the degree of pneumonic involvement varied. Histopathological findings were suggestive of bronchopneumonia. It was estimated that a single initially infected calf can contaminate a pasture with as many as 33,000,000 larvae.

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The Resistance of Milk Samples to the Activity of *Streptococcus Agalactiae* as Affected by Pasture

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KNOWLEDGE of factors that possibly may be involved in the resistance of dairy cattle to mastitis infections and satisfactory means for investigating this problem are rather limited. One approach has been to study the resistance of milk samples to the action of *Streptococcus agalactiae*.[2,5] The rations fed have been among the factors that seemed to be related to the differences encountered between samples. Milk samples from cows receiving relatively low grade hay, mostly timothy, as the sole roughage were compared for a limited period with those from cows fed forage-crop silage and alfalfa hay.[5] Acid production by the Streptococcus was the criterion used and this was greater in samples from the former than in those from the latter animals. The greater acid production in the milk from the annals fed timothy hay was interpreted to mean that the milk was less resistant to streptococcal activity. Further studies have been made of the resistance of milk samples from cows on different forages. The present report concerns examination of samples from animals on pasture.

EXPERIMENTAL PROCEDURE

Quarter milk samples collected weekly from three groups of dairy cows were compared during the pasture season of 1952. Prior to pasture they were fed hay, mostly alfalfa, forage-crop silage, and grain. The 17 cows in group 1 (6 Holstein-Friesians and 11 Jerseys) were grazed on bluegrass pasture for their roughage component and the 23 Jerseys, comprising group 2, were pastured on legume and brome grass. The 14 cows (7 Holstein-Friesians and 7 Jerseys) in group 3 were not on pasture but, for roughage, were fed mixed hay, mostly timothy. The milk samples were aseptically collected in the morning within two hours after the animals were milked. Using procedures similar to those previously described,[2] 10-ml. quantities of milk were exposed to the action of the Streptococcus. Each sample was placed in a tube containing bromcresol purple and was inoculated with the same strain of *Str. agalactiae* as the one used in the earlier work. After incubation for 24 hours, measurement of acid formation was accomplished by titrating each sample back to approximately the original bromcresol purple color, using 0.1N NaOH.

Average quantities of NaOH required to neutralize the acid during the month prior to going on pasture were the same for all three groups—2.4 ml. A marked loss in resistance to the formation of acid developed in samples from cows in groups 1 and 2 immediately after going on pasture. The change in the resistance of the milk samples occurred within a week in both pasture groups. It became more noticeable because the animals did not go on pasture the same week, the date being April 22 for the legume pasture, and May 1 for the grass pasture (graph 1). Average quantities of NaOH required for the first month on pasture were, for group 1, 3.1 ml. and 3.0 ml. for group 2 in contrast to 2.7 ml. for group 3 (graph 2). These figures represent an increase of 29.2 per cent for group 1 and 25.0 per cent for group 2 over the prepasture average but only 12.5 per cent for group 3.

Smaller groups of animals were available for similar study during the early part of the 1953 season and in 1954. The weekly average quantities of sodium hydroxide required are summarized (table 1) for samples collected from animals during the three weeks before pasture and during the first month on pasture in the three years. The figures for three weeks prior to pasture were used instead of four weeks, as this made it possible to use a similar period of time for all three years and to include a number of animals for which a fourth test result was not available. Four cows in the pasture groups in 1952 were omitted from this table because their time of freshening was too late for making three prepasture observations. Included also in the table is a summary of tests conducted during the same periods on other animals which did not go to pasture but which were fed dry feeds consisting only of hay and grain.

The changes from winter feed to pasture in 1953 and 1954 were not accompanied in the earlier weeks by as definite changes in the quantities of sodium hydroxide needed in the titrations as in 1952 even though the trends were similar. The work was done by the same people in 1952 and in 1953, which helped rule out the possibility that a human element was involved in the differences.

The data given in table 1 for the 20 cows fed hay and grain during the periods that the others

From the Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster, Animal Disease and Parasite Research Branch, Agricultural Research Service, U.S.D.A., cooperating.

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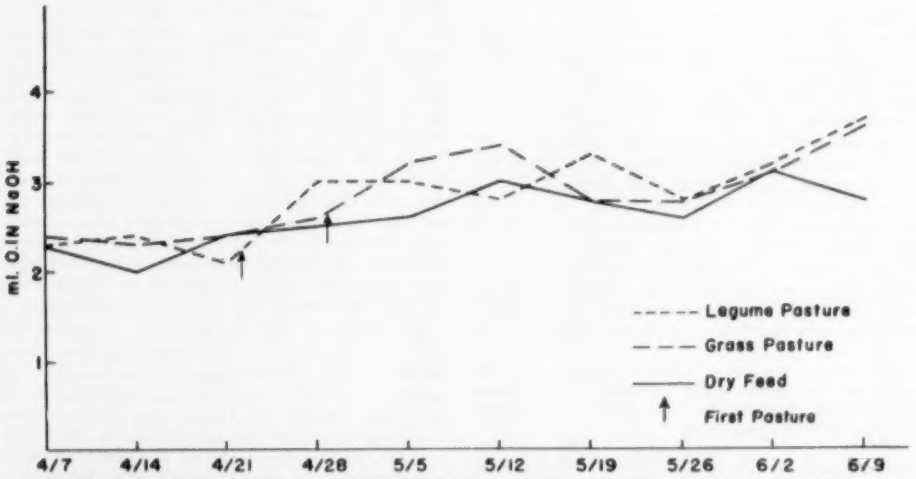
TABLE 1—The Average Quantity of 0.1N NaOH Required to Neutralize Acid Formed by Streptococcus Agalactiae in 10.0-ml. Milk Samples from Cows Changed from Dry Feed to Pasture

		Consecutive weekly tests (ml. 0.1N NaOH)										
		Before pasture				On pasture					Increase	
Year	No. of cows	3	2	1	(Av.)	1	2	3	4	(Av.)	(ml.)	(%)
1952	36	2.3	2.4	2.6	2.43	3.1	3.3	2.9	3.1	3.10	0.67	27.5
1953	22	2.8	2.7	2.7	2.73	2.9	3.1	3.3	3.2	3.15	0.42	15.4
1954	26	2.1	2.2	2.3	2.20	2.4	2.7	2.5	2.5	2.52	0.32	12.7
Av.	84	2.37	2.58	2.53	2.49	2.83	3.06	2.88	2.97	2.94	0.45	18.5
Average, dry feed cows (same periods), three years												
	20	2.38	2.72	2.83	2.64	2.78	3.08	2.88	2.66	2.85	0.21	8.0

were put out to pasture include 4 cows in 1953 and 2 in 1954, in addition to the 14 mentioned previously for 1952. The average titrations for the samples from these 20 head show an increase for the period covered by the four tests, during which the other cows first were on pasture, over the prepasture time. However, the increase was smaller and less abrupt than in the samples from the cows on pasture. Checking these animals provided somewhat of an additional control even though not an adequate one because most of them were taken off silage on or about the same time as the others went to pasture and samples from some animals restricted to a hay and grain diet tend to become less resistant to acid formation by *Str. agalactiae*.[2]

The differences in quantities of sodium hydroxide required to neutralize the acid in the milk samples during the prepasture and pasture feeding periods were subjected to statistical analysis. The procedure used was an analysis of variance[6] involving calculation of "F values" which provide expressions of the significance of differences when compared with tables of established values. The F value for the 1952 prepasture and pasture comparison was 27.15 where the 1.0 per cent level was 7.44; thus a high degree of significance was indicated. The significance was less for 1953 and 1954 when the F values were 5.2 and 4.82 with the 1.0 per cent and 5.0 per cent levels being 6.76 and 3.89, respectively. When the data from all three years were consolidated, a very significant F value was obtained, 31.7 with the 1.0 per cent level of probability 6.70. The increase during the same periods for the cows on dry feed was significant at the 5.0 per cent level of probability for the 14 animals in 1952 and at the 1.0 per cent level for the three years' total of 20 head with an F value of 8.3. Thus the increases in the quantities of NaOH required for samples from the pasture-fed animals were much more significant than those from animals on dry feeds.

A further increase occurred in the average quantities of NaOH required to neutralize the acid formed in the samples from both pasture groups in the second month (June) on pasture in 1952 (graph 2). A peak for the early pasture phase was reached during the second week of this



Graph 1—Average quantities of 0.1N NaOH required to neutralize the acid formed by Streptococcus agalactiae in weekly milk samples collected from cows fed dry feeds and others changed from dry feeds to pasture.

month. Monthly average quantities for both pasture groups were the same, 3.3 ml., which is a 37.5 per cent increase over the prepasture monthly average. A smaller average quantity, 2.7 ml., representing a 12.5 per cent increase was recorded for the dry feed group.

Comparable data were not available for 1953. In 1954 a trend rather similar to 1952, but less marked, was encountered during the same early pasture months. However, the peak was reached a few days sooner as it occurred at the end of May and beginning of June.

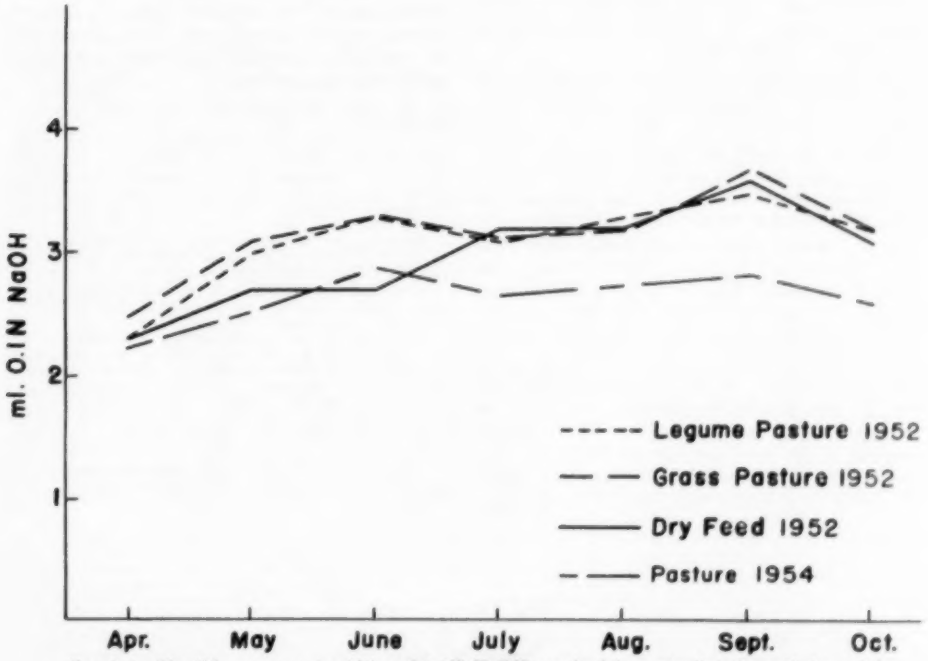
Reductions in the average quantities of NaOH required to neutralize the samples were recorded in the third month (July) for the groups of cows on pasture in both 1952 and 1954 (graph 2). During the same month in 1952 there was an increase in the average quantity needed for samples from group 3, the dry feed animals (graph 2). The figures for all three groups in July, 1952, were very nearly the same. They remained close together during the next three months, the quantities for the first two (August and September) were increased while the last (October) was a reduction from the previous month. The increase in the September averages of the two pasture groups in 1952 was caused, for the most part, by sharp rises early in the month when pastures were helped by August rains. Hay feeding began in the middle of September to support failing pasture, and silage feeding was started in mid-October when all three groups were given similar feed.

Data were available for a limited number of animals throughout the 1954 season. The results obtained in the summer and fall of 1954 differed from those of 1952 in that no marked increases occurred in the monthly average quantities of sodium hydroxide required to neutralize the samples (graph 2). In fact, a similar milk resistance level was maintained up to winter feeding time following the improvement in the middle of June.

Discussion

The loss of resistance of milk samples to acid production by *Str. agalactiae* when cows are changed to pasture parallels the rather frequently encountered clinical picture of mastitis associated with this organism. For example, Murphy [1] described instances in which considerable increases in mastitis occurred in a large herd during the earlier weeks it was on pasture. This happened in two different years of the several he had the herd under observation.

A number of herds in which many animals had mastitis at one time were encoun-



Graph 2—Monthly average quantities of 0.1N NaOH required to neutralize the acid formed by Steptococcus agalactiae in weekly milk samples collected from cows fed dry feeds and others on pastures.

tered over the years including several when conducting field control studies.[3] For instance, early in the season two neighboring herds were turned out to pasture into fields bordering on a river bank. Within a month there were eight cases of mastitis among the 15 cows in one herd and almost as high a proportion among the 23 cows in the other. When a single check of these herds was made, *Str. agalactiae* was isolated from almost all the affected animals.

Two other herds were brought to our attention early in August because both had encountered extensive trouble with mastitis during the preceding months. Eight cows with active cases and 13 others with histories of recent attacks were present in one herd of 32 animals. There were 19 affected animals out of 27 in the other herd. Again *Str. agalactiae* was present in milk samples from most of the animals. Faulty milking practices and mismanagement procedures commonly associated with mastitis problems were not present in these herds and all were owned and operated by experienced dairymen. Unexplainable herd problems, such as these, stimulated the search for methods that might provide some indication of relative differences in resistance to the disease.

In discussing the factors that might be involved in stimulating the attacks, Murphy[1] considered that they were caused either by a direct action of weather, as in chilling, or by an indirect action on the chemical composition of the forage. As weather temperatures were not unusually low it would seem more reasonable that the effect was indirect and through the feed consumed. This concept logically can apply to both the mastitis problem as observed by Murphy[1] and the milk resistance observations reported here because there appeared to be a relationship between the available pasture and the resistance level of the milk. The pasture varied with the weather so that the latter indirectly affected the resistance level. Rainfall was quite good in 1952. Pasture grass was relatively plentiful in late August and early September following August rains and resistance levels decreased. The opposite was true of the weather during the 1954 season, and resistance levels were maintained quite well. Thus, another point in common is the variation between years.

Further support for the concept that the effect was through the feed is provided by the silage feeding results[4] in which a feed change was associated with a loss in milk resistance.

SUMMARY

Weekly milk samples were obtained from cows just prior to and after the cows were turned on pasture and were tested for resistance to acid production by *Streptococcus agalactiae*. Samples collected in the weeks immediately after the feed change were less resistant than those obtained prior to the change. Partial recovery in resistance followed and this was maintained to the end of the summer in one year but, in another, further losses again occurred.

A paralleling situation was noted between the seasonal losses encountered in resistance of the samples from the experimental herds, and the onset in other herds of extensive mastitis problems associated with *Str. agalactiae*.

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The Resistance of Milk Samples to the Activity of *Streptococcus Agalactiae* as Affected by Feeding Silage

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RATIONS of alfalfa hay, silage, and grain are considered among the better ones for dairy cows judged on their nutritive value and on their ability to stimulate and maintain milk production. Preliminary studies[5] indicated that these rations were satisfactory for maintaining resistance, in milk samples, to acid production by *Streptococcus agalactiae* but the experimental period was short, being limited to a few weeks. The method used to determine resistance was based on the relative quantity of sodium hydroxide required to neutralize the acid formed by the Streptococcus when incubated in the milk samples.[5] A comparison of this method as a measure of resistance of milk samples with one based on increases in numbers of the streptococci in the samples failed to show a relationship.[3]

In some years, a trend toward losses in resistance of milk samples was observed when animals were first turned out to pasture.[4] This was particularly true in one season. Furthermore differences were observed, between years, in the level of resistance to acid production that could be maintained during the remainder of the pasture period. More gradual loss in resistance was encountered in samples from cows fed rations composed only of hay and grain.[2, 4]

EXPERIMENTAL PROCEDURE

Opportunities existed in 1951, 1952, and 1954 to study the occurrence of changes in this resistance when animals went on winter rations consisting of silage, hay, and grain. Herds maintained on different rations prior to the start of the silage feeding were included.

Resistance levels of the milk samples, to acid production by *Str. agalactiae*, were determined in the same manner as previously described.[6] Quarter samples were collected aseptically within two hours after the morning milking on one day each week. Subsequently 10-ml. quantities of each sample were inoculated with a strain of *Str. agalactiae* with addition of bromcresol purple as is done in the Hotis test.[1] Following incubation at 37 C. for 24 hours, the samples were titrated back to approximate their original purple color using 0.1N NaOH.

Effect of Addition of Silage to a Hay and Grain Ration.—A loss in this resistance was observed, in 1951, when corn silage, at the rate of approximately 30 lb. per day per animal, was added to a ration of grain and medium quality timothy hay mixed with a little clover, fed to 7 Holstein-Friesian cows which had been without silage or pasture for at least 18 months. Although the herd contained more than twice this many animals, these 7 were the only cows in production continuously through this period of feed change. When the average quantities of sodium hydroxide used in the titration of milk from these 7 cows was recorded (table 1), for the four weekly tests before and after starting the feeding of silage, it was seen that relatively large increases in sodium hydroxide, indicating lowered resistance of the samples, were required during the weeks immediately following the beginning of silage feeding, on Nov. 29, 1951. The higher readings persisted throughout December then gradually lessened in January. They continued to drop to the extent that in February the average reading was lower than in November. The averages for each of the 7 cows

TABLE 1—The Average Quantity of 0.1N NaOH Required to Neutralize Acid Formed by Streptococcus Agalactiae in 10.0-ml. Milk Samples Collected Once Each Week from 7 Cows on Addition of Corn Silage to a Hay and Grain Ration

Weekly Averages of 0.1N NaOH (ml.)							
Presilage				Silage			
4	3	2	1	1	2	3	4
3.8	3.4	3.5	3.4	4.0	4.3	4.5	4.6

Monthly Averages of 0.1N NaOH (ml.)			
Presilage	Silage		
November	December	January	February
3.52	4.35	4.16	3.1

From the Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster (Pounden and Frank) and the Animal Disease and Parasite Research Branch, Agricultural Research Service, Beltsville, Md. (Brown and Scherer).

Grateful acknowledgment is made for the assistance of the Department of Dairy Science, Ohio Agricultural Experiment Station, Wooster, and of the Dairy Production Branch, Agricultural Research Service, Beltsville, Md.

TABLE 2—The Average Quantity of 0.1N NaOH Required to Neutralize Acid Formed by Streptococcus Agalactiae in 10.0-ml. Milk Samples Collected Once Each Week from Cows Changed from Pasture to Rations of Hay, Silage, and Grain.

Number cows	Weekly Averages of 0.1N NaOH (ml.)								Monthly average difference (ml.)
	Presilage				Silage				
	4	3	2	1	1	2	3	4	
1952									
35 cows	3.3	3.5	3.3	3.2	2.9	2.8	2.8	2.7	
			Av. 3.3				Av. 2.8		—0.5
1954									
10 cows	2.8	2.7	3.2	3.1	2.4	2.8	2.5	2.6	
			Av. 3.0				Av. 2.6		—0.4
Total									
45 cows	3.2	3.3	3.3	3.2	2.8	2.8	2.7	2.7	
			Av. 3.25				Av. 2.75		—0.5

increased during December and each was involved also in the reduction that came about by the end of January. The December average of 4.4 ml. represents a 25 per cent increase over the November figure of 3.5 ml.

Average sodium hydroxide figures for the herd as a whole showed similar trends during the same months even though changes occurred because of the beginning and ending of lactations. The monthly average for the herd in December was higher (4.6 ml.) than in any month during the 21 months the herd was under study while the February average was equal to the lowest (3.1 ml.) or most resistant average recorded.

Effect of Addition of Silage to a Pasture and Hay Ration.—In the fall of 1952, 35 Jersey cows were changed from a ration of alfalfa-blue grass pasture, alfalfa hay, and grain to one of forage-crop silage with the hay and grain. Following this ration change, the quantities of sodium hydroxide required to neutralize the acid formed in the milk samples by *Str. agalactiae* became somewhat smaller which indicated a moderate increase in resistance of the samples (table 2). No change was observed, however, when corn silage was substituted two weeks later.

Another group of animals, 3 Jerseys and 7 Holstein-Friesians, were studied in the fall of 1954, for four weekly tests prior to and after the change over from pasture to the feeding of corn silage (table 2). While the figures show a temporary gain in resistance of the milk samples for the first weekly test after the change in feed, the results over the eight-week period do not indicate any definite trend.

Resistance Levels on Alfalfa Hay, Forage-Crop Silage, and Grain.—Quarter milk samples from 12 Jerseys and 5 Holstein-Friesians were examined in the fall and winter months, 1952–1953, for resistance each week beginning immediately after the animals freshened and while they were on a ration of alfalfa hay, forage-crop silage, and moderate grain feeding. In the months immediately preceding fall freshening, 10 of the cows were on pasture and grain feeding while the other 7 were fed mixed hay of medium quality with grain. During the period when the relatively good quality winter ration was being fed, it was possible to obtain consecutive observations on the quantities of sodium hydroxide required per sample for each lactation month for 10 cows for 8 months, on 2 for 6 months, 3 for 5 months, and 2 for 4 months (table 3).

TABLE 3—Daily Pounds of Milk and Monthly Average Quantities of 0.1N NaOH Required to Neutralize Acid Formed by Streptococcus Agalactiae in 10.0-ml. Milk Samples Collected Weekly from Cows on a Continuous Ration of Alfalfa, Forage-Crop Silage, and Grain

	Lactation Months (Av. 10 cows)							
	1	2	3	4	5	6	7	8
NaOH (ml.)	3.2	3.1	2.8	3.0	3.1	3.0	2.9	3.0
Daily milk (lb.)	39.3	38.5	35.0	31.1	28.5	25.7	23.8	24.1
	Average All Cows							
No. of cows	17	17	17	17	15	12	10	10
NaOH (ml.)	3.1	3.1	2.8	3.0	3.1	3.0	2.9	3.0

The figures indicate that the average level of resistance of the milk from these animals to the action of the Streptococcus was relatively good and was maintained throughout the period in which this ration was fed. A check of the individual monthly averages showed that resistance levels were very well maintained in samples from 12 of them, that resistance was lost to a moderate degree in those from 2, to a slight degree from 1, and for a temporary period in those from 2 others during their fourth and fifth months. The differences in the feeding program during the months prior to freshening did not appear to influence the results when the figures of the two groups were compared.

Average daily milk weights for the 10 cows, 3 Holstein-Friesians and 7 Jerseys, also are included in table 3. The milk production of the cows in this group was reasonably good. The heaviest monthly average for any individual was 63.8 lb. per day for a Holstein-Friesian and 46.1 lb. per day for a Jersey. All of them maintained production within reasonable limits to the end of the period of observation.

Milk samples were obtained from a limited number of animals being fed forage-crop silages as the only roughage or as a major part of this component of the ration. These were collected once each week during a 17-week observation period. To facilitate summarizing the data (table 4), they were averaged in four periods of approximately a month each, there being three periods of four weeks each, and the last period of five weeks. During the first four-week period, the roughage for all cows consisted of equal parts of forage-crop silage and alfalfa hay based on dry matter weights. Following this, the roughage consisted of silage only for 3 cows, 80 per cent silage for 2 cows, while the remaining 3 cows were continued on equal parts of roughage and silage.

The results of this feeding program can only be

TABLE 4 — Average Quantities of 0.1N NaOH Required to Neutralize Acid Formed by Streptococcus Agalactiae in 10.0-ml. Milk Samples Collected Once Each Week from Cows Receiving Forage-Crop Silage as a Major Component of Their Roughage Ration

No. of cows	Roughage proportion		Months			
	Silage	Hay	1	2	3	4
3	100	0	1.7	1.9	2.0	2.0
2	80	20	3.0	2.9	2.7	2.3
2	50	50	2.3	2.2	2.7	2.2
7 cows (av.)			2.24	2.27	2.40	2.14

considered as an indication because the number of animals was so small, but they are rather similar to those obtained in the larger and more lengthy experiment mentioned above. The forage-crop silage was apparently associated with a relatively good resistance by the milk samples to the action of *Str. agalactiae* even when fed as the only roughage.

Discussion

The difference in the responses, to the activity of *Str. agalactiae*, of milk samples from cows having silage added to drylot feeding compared to those which had been on pasture, probably can be explained easiest by the difference in the severity of the feed change. The loss in resistance, observed in milk samples from the animals previously on dry feeds alone, appears to be similar to the sudden losses encountered in milk samples from cows changed from winter feed to pasture.[4]

Relatively high levels in resistance were maintained by milk samples from cows on rations containing silage, either forage-crop or corn. This was true of the eight-month observation, the longest period covered by this report. Similar results for a shorter period had been obtained in earlier studies.[5] When silage comprised the whole or a major part of the roughage fed, the milk samples also retained a satisfactory level of resistance during the limited period of observation. In the only instance in which losses in resistance occurred on change to silage feeding, resistance levels in the milk samples gradually changed back and became better than before.

If the results of this test reflect the suitability of the environment within udders for *Str. agalactiae*, it is possible that the feeding programs in recent years have had something to do with the reduction in the incidence of *Str. agalactiae* infections. Much stress has been placed on use of better quality and greater quantities of roughages in dairy cattle feeding. Use of forage-crop silage has received considerable emphasis, if not the major emphasis, in the programs stressed in this and neighboring states. Although experience with experimental and farm herds has been very limited, it has provided support for the idea that the feeding program may have some effect in the control of mastitis associated with this organism.

Variations in results with this technique sometimes are encountered between samples from different quarters on the same cow, in samples from the same cow at different times, and between cows in the same environment. It can not be stated that these variations always reflect actual variations in resistance of the samples. While it is logical to hesitate to rely on the results of so few tests, the information which has been or may be obtained by these methods can be used as a guide in attempts to control mastitis in herds where this problem can not otherwise be adequately explained.

Summary

Fresh milk samples, obtained from cows prior to and during the period of feeding of silage, were tested for resistance to the activity of *Streptococcus agalactiae*. This was accomplished by inoculating with the organism and measuring the relative acid production 24 hours later by titration with sodium hydroxide.

Resistance of the milk samples was lowered, for a few weeks, when corn silage was added to the hay and grain ration which had been fed continuously for a year and a half. Resistance was retained or improved slightly when cows which had been on pasture all summer were given forage-crop silage as part of their ration in the fall.

A relatively good level of resistance was maintained during an eight-month observation while the cows were fed rations of forage-crop silage, alfalfa hay, and grain. Satisfactory levels also were maintained during a more limited period while the cows were fed forage-crop silage as their sole or main source of forage.

The possibility is suggested that the feeding, in recent years, of better quality and greater quantities of roughages may have helped reduce the incidence of *Str. agalactiae*.

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An Agent Enhancing Listeriosis of Sheep

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Listeria monocytogenes usually has been accepted as the causative agent of "circling disease" of sheep since Gill's report [1] in 1931. Listeria can be isolated from most field cases of listeriosis if the appropriate technique is used;[2] however, fulfillment of the second of Koch's postulates, that is, production of the disease in sheep by inoculation with a culture of the microorganism has been more difficult.

This report describes experiments in which listeriosis was regularly produced in sheep by simultaneous exposure to *L. monocytogenes* and a virus-like agent. The agent was isolated during a natural outbreak of listeriosis.

Different routes of inoculation of sheep with Listeria have been tried. Intracerebral exposure of sheep is usually fatal in 24 to 72 hours.[3-7] Gill [8] reported encephalitis and death in seven days after injection of Listeria into the carotid artery of 1 sheep and Jungherr [9] reported death in 48 hours in 1 sheep similarly exposed. Olafson [5] stated that "inoculation into the carotid artery destroys sheep about as quickly as intracerebral inoculation." Biester and Schwarte [3] observed only a rise in body temperature followed by recovery after intracarotid exposure. Olson *et al.* [10] reported death of 19 sheep six to 63 days after intracarotid exposure. In another series of experiments, 2 of 8 sheep inoculated by way of the carotid artery died 22 to 29 days later.[11] There was also a distinct difference in pathogenicity between two strains of Listeria for comparable test sheep.

Intravenous exposure was usually followed by an increase in temperature and marked neutrophilia.[12] Encephalitis and death did not usually occur [5, 7, 11, 13] although Graham *et al.* [4, 14] reported encephalitis and death in 13 of 14 sheep intravenously exposed to Listeria. Olson and co-workers [10] caused death of 10 of 37 sheep 8 to 15 months old by intravenous inoculation.

Subcutaneous injection of *L. monocytogenes* is usually without effect.[3, 5, 12] Biester and Schwarte produced nervous symptoms and death in 1 sheep after a series of 15 subcutaneous and intramuscular injections.[3] Olson *et al.*[10] attempted to immunize a large number of sheep by two subcutaneous inoculations of Listeria during an outbreak of listeriosis. Subsequently, listeriosis was more prevalent in the sheep so treated than in the untreated controls, indicating that subcutaneous injection can cause infection in a few animals.

Irregular results have been reported following intranasal exposure with *L. monocytogenes*. Gill [8] observed nervous symptoms and death in 2 of 3 sheep, seven and eight days after intranasal exposure. Jungherr [9] was unable to produce listeriosis in 1 sheep by intranasal exposure. Olafson [5] reported negative results in trials with 20 animals. Olson *et al.*[11] failed to produce listeriosis in 3 sheep exposed intranasally, although in 1 there was a transient bacteremia on the second and third days after inoculation. Osebold and Inouye [15] reported an elevation of temperature and increase in neutrophils in 3 lambs and in 1 ewe exposed intranasally but there was no clinical evidence of disease in the central nervous system.

Other methods of experimental exposure reported in the literature include intrarumen,[5, 11-13, 16, 17] conjunctival instillation,[5] exposure of the vaginal mucosa and of the submucosa of the prepuce,[16] and injection into the median artery.[12] Typical encephalitis did not occur in the sheep thus exposed.

Asahi and Hosoda [16] recently reported the development of typical listeriosis in 4 of 11 goats which received Listeria in the submucosa of the lip. Two of 8 goats given rough feed contaminated with Listeria developed the disease. The authors suggested that the rough feed produced small wounds in the oral mucosa through which Listeria gained entrance.

From this brief review of the attempts to produce listeriosis in sheep, it appears that experimental production of the disease has been obtained consistently only by intracerebral and intracarotid exposure to the Listeria organism. Since these routes are unnatural, other factors have been considered which may render the organisms more virulent or the animals more susceptible.

Olafson [5] observed that most outbreaks of listeriosis occur in flocks fed silage. This observation has been confirmed by other workers.[14, 18] The relation of silage to listeriosis was put to trial by Olson *et al.*[11] but the experimental feeding of silage had no demonstrable effect on the development of listeriosis.

Olson and co-workers [20] injected Listeria intravenously into 8 sheep together with a suspension of brain and lung from a calf which died with malignant catarrhal fever. Only 3 of the 8 animals died and *L. monocytogenes* was isolated from their brains and visceral organs. This result was considered not significantly different from what

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might be expected after intravenous exposure to Listeria only.

MATERIALS AND METHODS

Listeriosis-Enhancing Agent (LEA).—An outbreak of listeriosis occurred in a flock of sheep during the winter of 1953. An attempt was then made to study the effect of feeding silage in a presumably infected environment. Eighty lambs from a flock with no evidence of listeriosis were moved to the environment of the infected flock and divided into two equal groups. One group was fed silage from the same silo which supplied the sheep involved in the outbreak of listeriosis. The other group received no silage. Rectal temperatures of the 80 lambs were measured daily. Whenever an animal had an elevated temperature, leukocytes were counted.

Listeriosis did not develop in any of the lambs. No elevations of temperature were noted in the 40 lambs which did not receive silage. However, 9 of the 40 lambs fed silage had elevated temperatures (up to 106 and 108 F.) which lasted for two to three days. No symptoms other than a variable degree of absolute neutrophilia accompanied the fever. Two of the 9 animals were killed during the period of fever and portions of the visceral organs and central nervous system examined for bacteria. Since no pathogenic organisms were recovered, it was thought that some viral agent might be the cause of the disturbance. One of the 9 lambs (lamb 329) was bled when its temperature was 108 F. The blood was defibrinated by shaking with glass beads and stored for future use. Tryptose broth and tryptose agar mediums inoculated with the blood remained sterile.

Two milliliters of this defibrinated blood, kept for two months at —35 C., was injected intravenously into each of four 7-month-old lambs. Temperatures of 105.8 to 106 F. occurred after approximately six hours in 3 of them. The fever was of short duration and was accompanied by an increase in neutrophils. No other symptoms were noticed. Blood was obtained aseptically from lamb 123 when its temperature was 105.7 F. This was placed into the allantoic cavity of 10-day-old chicken embryos. The eggs were incubated at 35 C. for five days, then killed by chilling at 5 C. for a few hours. The allantoic fluids were harvested and used to make ten further passages by the allantoic sac route of inoculation. No mortality or gross pathological changes were observed in the chicken embryos.

Serial passages by inoculation of the yolk sac were started with allantoic fluid from the tenth passage. Six- to 8-day-old chicken embryos were used. The inoculated embryos were incubated at 35 C. for five to seven days, then killed by chilling. A portion of either nonwashed yolk sac membrane or the embryo was ground and suspended in approximately ten volumes of saline solution and used as inoculum for the serial transfers. No significant mortality of the chick embryos occurred. Most of the embryos, however, had some subcutaneous congestion, especially in the region of the neck and feet, and small hemorrhages in different areas of the body. The chorioallantoic membrane was occasionally edematous.

Twelve eggs were inoculated with material from the sixth yolk sac passage. After the usual incubation, the embryos were harvested and stored at —35 C. This material when ground and suspended in physiological salt solution constituted the listeriosis-enhancing agent (LEA) inoculum for most of the experimental exposures of sheep described in this report.

This material was also used to inoculate cultures of malignant human epithelial cells (strain HeLa, Gey *et al.*[21]). The tissue culture technique described by Syverton and co-workers[22] was followed with minor modifications. The cultural fluid of the fifth serial transfer was used as inoculum for 2 sheep.

Blood from lamb 123, stored at —60 C. for 18 months, was also used as inoculum for 2 sheep.

Bacterial Agent.—*Listeria monocytogenes* strain 1501–2, isolated in 1951 from the medulla oblongata of a sheep in an outbreak of listeriosis, was used throughout the experiments. For intravenous exposure, a dose of 0.1 ml. of a 24-hour tryptose broth culture per pound of body weight was employed. Such cultures contained approximately 2×10^9 viable microorganisms per milliliter. Intranasal exposure was made by spraying about 1 ml. of a 24-hour tryptose broth culture deeply into both nostrils of the sheep using a DeVilbis No. 150 atomizer. The animals so treated frequently sneezed during or shortly after exposure, expelling part of the fluid which had been introduced into their nose. Therefore, no reasonable estimate can be made of the number of microorganisms which the animals actually retained.

Techniques.—Most of the experimental animals were lambs 1 year or less of age. Two adult ewes were included in the group exposed intravenously and one 18-month-old sheep in the group exposed intranasally.

Both total and differential counts of white blood cells were made prior to exposure of the sheep and several times on the day of exposure. Daily counts were made for the first seven days after exposure and at longer intervals thereafter. The rectal temperature was measured at the time white blood cell counts were made.

Blood of the sheep was examined for Listeria by culture two or three times on the day of exposure and daily thereafter until it was negative for three consecutive days. Four milliliters of blood were drawn from the jugular vein with a sterile syringe and distributed in four tubes of citrated tryptose broth.

Bacteriological examinations were made of the viscera and central nervous system. Portions of cerebrum, cerebellum, medulla oblongata, brachial and lumbar regions of the spinal cord, liver, spleen, kidney, lung, heart, and prescapular and mesenteric lymph nodes were ground in Ten Broeck tissue grinder and placed in two tubes of

tryptose broth. Also occasionally examined were cerebrospinal fluid, the root of the trigeminal nerve, the infraorbital, lingual and maxillary branches of the trigeminal nerve, the sciatic nerve, the pharyngeal, parotid, and submaxillary and prefemoral lymph nodes. Routine histological examinations were made of most of the tissues that were cultured.

TABLE 1—Results of Exposure of Sheep to Listeria Monocytogenes in Combination with Listeriosis-Enhancing Agent (LEA) or Alone

	Mode of exposure	Number exposed	Listeriosis
1	Listeria IV + LEA	7	7
2	Listeria IN + LEA	7	6
	CONTROLS		
	Listeria IV	21	6
	Listeria IN	4	0
	Listeria IN + tissues devoid of LEA	10	1

IV—intravenously; IN—intranasally.

Two methods were followed in exposing sheep to Listeria and LEA. In method 1, Listeria were inoculated intravenously and the LEA was inoculated both intravenously (2 ml.) and intranasally (1 ml.). This method will be referred to as "intravenous" for distinction from method 2 in which equal parts of Listeria culture and LEA were mixed together and inoculated intranasally. Control animals did not receive LEA. They were given Listeria either alone or, in some instances, with a suspension of tissues from normal chicken embryos. Several attempts were made to demonstrate a listeriosis-enhancing effect with suspensions of lymph node tissue from cattle which had died with suspected viral infections. In those trials where the results were clearly negative, the information was used as control data.

Serum neutralization of LEA was attempted using serums from 2 sheep which had been hyperimmunized by repeated intravenous injections of material known to contain LEA.

RESULTS

Seven susceptible animals received Listeria intravenously with LEA (exposure method 1, table 1). All died, and *L. monocytogenes* was recovered from their central nervous systems. Seven susceptible sheep received organisms with LEA intranasally (method 2, table 1). Six of the animals developed listeriosis and 1 survived.

Twenty-one susceptible sheep were inoculated intravenously with Listeria but did not receive the LEA. Six of these developed listeriosis and 1 survived the disease. Four sheep were exposed intranasally to Listeria only and none developed listeriosis.

Ten sheep were exposed intranasally to Listeria with normal chicken embryo suspension or lymph node material from cattle. One of these which received *L. monocytogenes* and normal chicken embryo material developed listeriosis in 28 days and died ten days later. Thus about 29 per cent (6 of 21) of the control sheep (those which did not receive LEA) inoculated intravenously developed listeriosis and about 7 per cent (1 of 14) of the controls exposed intranasally died.

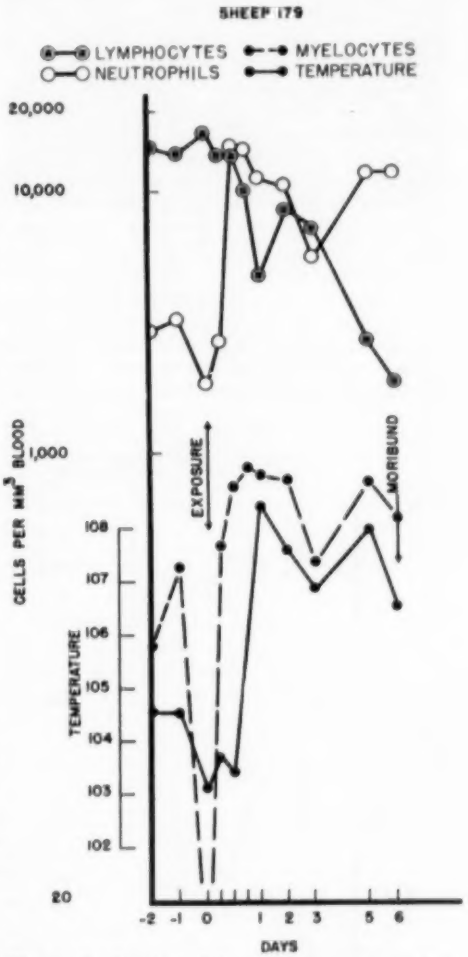


Fig. 1—Leukocyte and temperature response of a sheep which received Listeria intravenously with listeriosis-enhancing agent and died six days later. Note the elevation of temperature and increase of neutrophils with myelocytes following exposure. Lymphocytes dropped to a low level prior to death.

The sheep exposed intravenously to LEA and Listeria had no clearly defined incubation period for there was an immediate reaction of leukocytes, fever, (fig. 1) and general depression which continued until death. One exception to this was sheep 649, an adult ewe which developed nervous symptoms 33 days after exposure.

After intranasal exposure to Listeria and LEA there was usually an initial febrile and neutrophil reaction which subsided and returned with the onset of nervous symptoms (fig. 2). The symptoms were variable but included lameness, torticollis, drooping of an ear, paralysis of lower jaw, and sometimes complete inability to stand. Most affected animals had a thick, tenacious, grey-white discharge of mucus from the nostrils (fig. 3). The incubation period (interval from intranasal exposure to development of nervous symptoms) was fairly uniform, varying from 18 to 22 days. *Listeria monocytogenes* was isolated from organs or tissues of all the experimental sheep which died. With only 1 exception, the animals exposed intravenously had Listeria in both viscera and the nervous system whereas the sheep exposed intranasally had Listeria in only the central nervous system (table 2).

Two ewes which received Listeria and LEA intravenously reacted similarly to the lambs of the same group, except that 1 of the sheep (sheep 649) developed listeriosis after a long incubation period and was killed on the thirty-sixth day.

Significant gross pathology was noted only in those sheep which received Listeria intravenously and died within eight days after exposure. In these animals, numerous small foci were scattered throughout the liver. Three of the sheep receiving Listeria with LEA had small microscopic lesions in the liver at necropsy 5, 18, and 22 days after exposure. The lesions consisted of focal accumulations of neutrophils (polymorphonuclear leukocytes) and polyblasts (lymphocytes and histiocytes) some of which were undergoing degenerative changes. Necrosis was not confined to cells in the center of the mass but was rather distributed throughout the lesion (fig. 4). These were located in the liver lobule without particular association with the periportal area or central vein.

The group receiving Listeria plus LEA

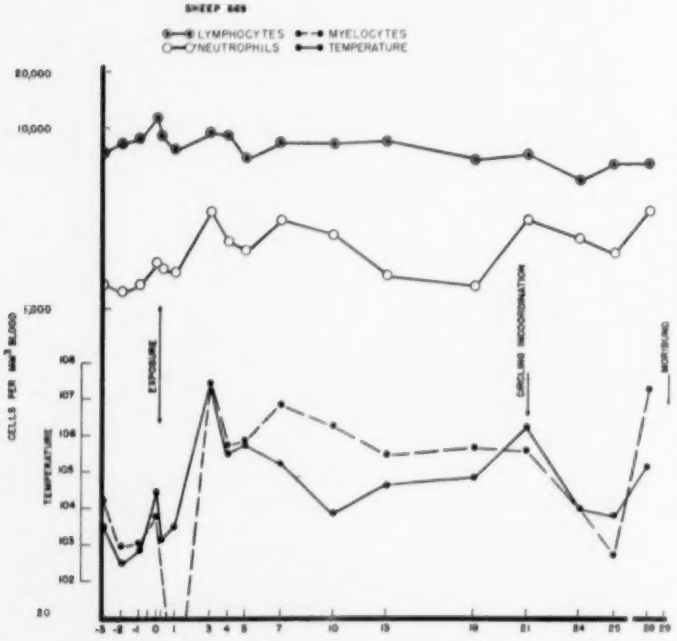


Fig. 2—Leukocyte and temperature response of a sheep which received Listeria intranasally with listeriosis - enhancing agent. Note temperature elevation and response of neutrophils three days after exposure with a trend toward a normal level until the twenty-first day when the first signs of brain disturbance were seen. Lymphocyte levels remained quite constant.

intranasally consistently had relatively marked lesions in the brain stem consisting principally of polyblasts which usually localized around blood vessels. Some neutrophils and polyblasts were scattered diffusely in the brain stem (fig. 5). In some cases, the infiltration was more marked on one side of the brain stem than the other. Similar lesions were observed in several cases at the base of the cerebellum (fig. 6). There were lesions of the lumbar portion of the spinal cord in three cases of listeriosis produced by the organism with LEA. In one case a neutrophilic infiltration extended into the nerve roots (fig. 7). In the same section, there was also marked meningitis (fig. 8). The lesions in the spinal cord were perivascular infiltrations with polyblasts, which seemed to extend from the meninges, and some areas of infiltration with neutrophils and local edema (fig. 9). The trigeminal nerve root was examined in 4 of the sheep receiving Listeria plus LEA intranasally, and in 3 there was a definite polyblastic infiltration extending into the root of the trigeminal nerve. The microscopic lesions in these sheep were quite similar to what has been observed in natural cases of listeriosis.

Three of the sheep which received Listeria with LEA intranasally had an increase of nuclei in the glomeruli of the kidney. A similar reaction was seen in 1 sheep which received only Listeria intranasally so the significance of the reaction was not clear.

There was a general correlation between lesions and positive bacterial findings in the experimental animals. The association of the lesions and successful isolation of Listeria was more like the natural disease in those sheep which received Listeria and LEA intranasally than in the other combinations of exposure.

Fig. 3—Nasal discharge and paralysis of right ear 23 days after intranasal exposure to Listeria and listeriosis-enhancing agent.

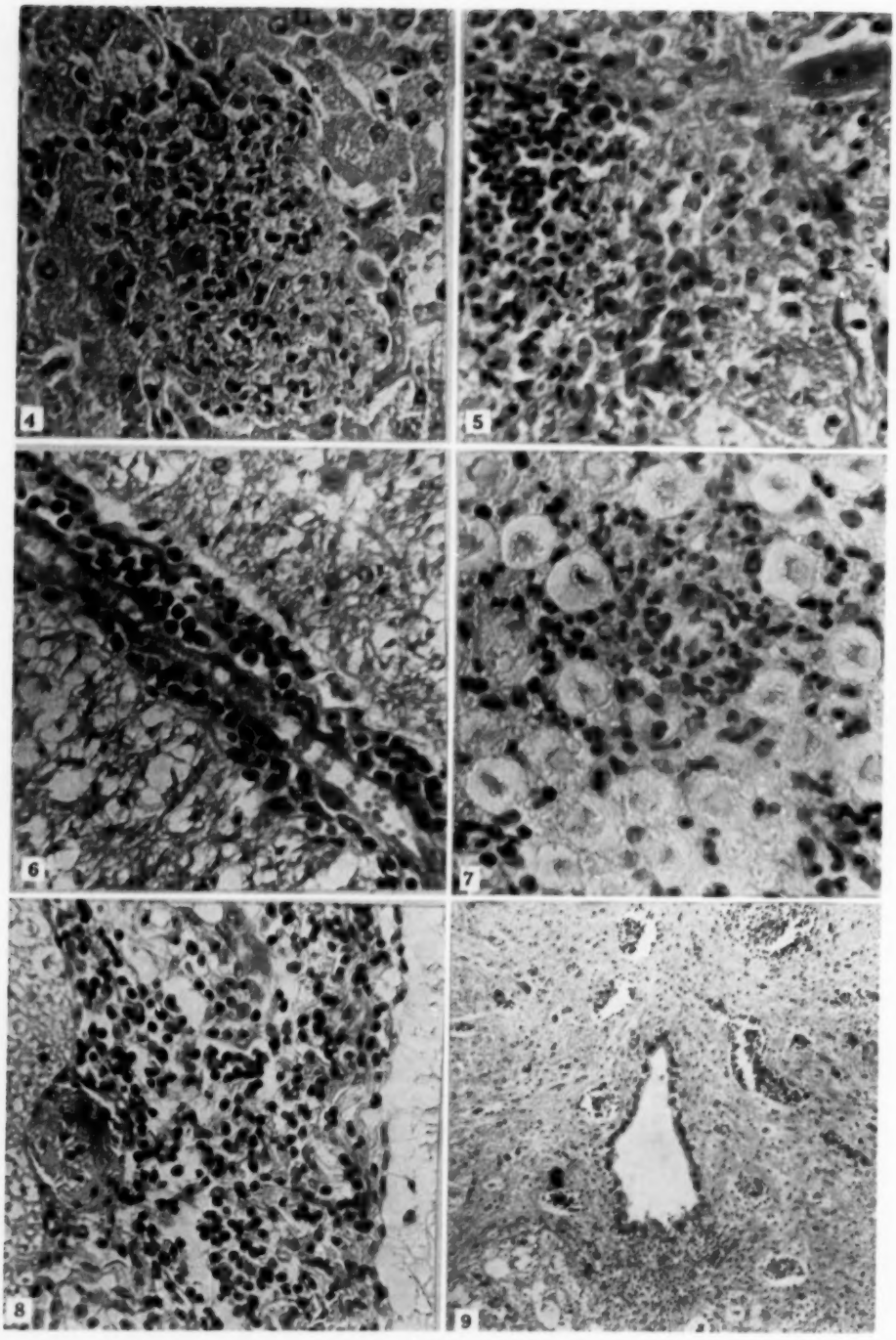
Discussion

Apparently LEA plays a definite role in aiding the entrance of Listeria and in the pathogenesis of the infection. For example, 6 of 7 sheep which were exposed by intranasal inoculation with a mixture of Listeria and LEA developed listeriosis. The incubation period of about three weeks following intranasal exposure and before development of nervous symptoms was quite uniform. The experimentally produced disease was very similar to the disease observed in natural outbreaks in sheep. By contrast, 4 sheep were given only Listeria intranasally and did not develop listeriosis. An additional group of 10 control animals received Listeria with tissues devoid of LEA and only 1 developed listeriosis. Other workers [5, 9, 11] have reported negative results from intranasal

TABLE 2—Bacteremia, Survival Time, and Distribution of Listeria in Sheep Which Died Following Exposure to Listeria Monocytogenes Alone or in Combination with Listeriosis-Enhancing Agent (LEA)

Method of exposure	No. exposed	No. died	Bacteremia: First detected	Bacteremia: Ave. duration (days)	Ave. survival (days)	Listeria isolated: CNS	Listeria isolated: Viscera
1 Listeria IV + LEA	7	7	10 min.	6	13	6	7
Listeria IV	21	5	10 min.	5	14	4	4
2 Listeria IN + LEA	7	5	0	0	25	5	0
Listeria IN devoid of LEA	10	1	5th day	1	38	1	0

IV—intravenously; IN—intranasally; CNS—central nervous system.


4
5
6
7
8
9

exposure. Gill [8] reported that 2 of 3 sheep exposed intranasally developed listeriosis. Osebold and Inouye [16] gave Listeria intranasally to 2 sheep then killed them six and 15 days later. Nervous symptoms did not develop but Listeria were recovered from the olfactory bulb of 1 and the medulla oblongata of the other.

When Listeria were given intravenously, about one-fourth of the sheep died with listeriosis. Other investigators obtained essentially similar results from intravenous exposure.[5, 7, 10-13] When LEA was given to the sheep which received Listeria intravenously all of the animals died. LEA was given both intravenously and intranasally to sheep, so there was no evidence of the relative importance of intravenous and intranasal exposure of LEA.

In some additional experiments, a small number of Listeria were given intravenously in combination with LEA. Two lambs survived a single dose of Listeria with LEA and showed no evidence of listeriosis. Two other lambs and 2 ewes were repeatedly given small doses (for example, 1 animal received eight small doses of Listeria and a single intravenous inoculation with LEA during a four-day period). These 4 animals also remained negative. Thus, a relatively large dose of Listeria was necessary to demonstrate the effect of LEA with intravenous inoculation. The disease produced in sheep by intravenous exposure to Listeria and LEA was somewhat different from the natural disease. In the naturally occurring disease, the organisms are usually confined to the central nervous system,[10] whereas in animals exposed intravenously, Listeria were found generally distributed in the visceral organs as well as in the central nervous system.

Some observations have been made on the listeriosis-enhancing agent. It has been propagated serially in the chicken embryo by inoculation into both allantoic sac and yolk sac. The agent has also been propagated in tissue culture with HeLa cells through five passages. Two sheep inoculated intranasally with Listeria and material of the last tissue culture transfer developed listeriosis. The agent contained in blood from sheep 123 was found active after being stored at —60 C. for 18 months.

Filtration trials with LEA have not been conducted. The agent can proliferate in but not cause death of chicken embryos and cultures of HeLa cells. At this time, its existence can be demonstrated only by production of listeriosis of the central nervous system upon simultaneous inoculation with Listeria into sheep.

Other animals in addition to sheep were exposed to LEA, alone or in combination with Listeria. A 1-month-old calf was given a mixture of Listeria culture and LEA in the prescapular lymph node and intranasally. Two 3-month-old pigs were exposed intravenously to both Listeria and LEA and one 3-month-old pig received LEA only intravenously. Twenty-four 1-

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Legends for Illustrations on Opposite Page

Fig. 4—Focal lesion in liver of an 8-month-old lamb which died six days after it was given Listeria intravenously. Note the mixture of neutrophils and polyblasts, many of which are degenerating. x 360.

Fig. 5—Edge of a localized accumulation of neutrophils in medulla oblongata of a 15-month-old sheep which died 20 days after it was given Listeria and listeriosis-enhancing agent intranasally. Listeria were isolated only from this portion of the brain. Some polyblasts are also present and a moderate proliferation of glial cells. x 380.

Fig. 6—Longitudinal section of blood vessel at base of cerebellum from a 5-month-old lamb which died 33 days after intranasal exposure to Listeria. Note the accumulation of polyblasts about the vessel without infiltration of adjacent brain substance. x 380.

Fig. 7—Nerve root from lumbar cord of an adult ewe (649) which developed nervous symptoms 33 days after it received Listeria and listeriosis-enhancing agent intravenously. There were marked lesions in the lumbar cord and the focal accumulations of neutrophils can be seen in the field illustrated. Axons appear in the cross section. x 470.

Fig. 8—Meningitis about cord in lumbar region from same animal whose lesions of the nerve root are shown in figure 7. The subarachnoid infiltration consists principally of polyblasts. Spinal cord is at the right edge of the illustration and the nerve root at the left edge. x 330.

Fig. 9—Cross section of neural canal and adjacent portion of lumbar cord from a 6-month-old lamb which died 23 days after intranasal exposure to Listeria and listeriosis-enhancing agent. There is a diffuse infiltration with neutrophils near the neural canal, at the bottom of the picture, and edema in the nearby white matter. Perivascular infiltrations with polyblasts are evident in upper part of photo. x 88.

week-old chickens were exposed intravenously to both Listeria and LEA, and 4 received LEA only. Two rabbits received LEA intravenously, and 2 by intradermal inoculation. Two mice were inoculated intradermally with LEA and 4 were exposed intranasally. Only those animals receiving Listeria showed evidence of illness and the symptoms were those which usually follow exposure to Listeria organisms only.

Several unsuccessful attempts were made to develop hemagglutination or complement-fixation tests for the LEA. Attempts to obtain neutralization of LEA with serum from sheep previously exposed to LEA gave inconclusive results.

A search should be made for the existence of a listeriosis-enhancing agent in other natural outbreaks of the disease in cattle and sheep. If such can be found, its action and characteristics should then be compared with the agent described in this report. The possible association of LEA with silage will require further study.

SUMMARY

A nonbacterial listeriosis-enhancing agent (LEA) was isolated from a sheep kept on the premises where an outbreak of listeriosis had occurred. A listeriosis-enhancing agent given to sheep in combination with *Listeria monocytogenes* resulted in listeriosis in nearly all sheep thus exposed. Intranasal exposure of sheep to Listeria and LEA produced clinical symptoms of listeriosis in 18 to 22 days and subsequent death after a relatively few days. Listeria were confined to the central nervous system. An infection different from natural listeriosis was produced by intravenous administration of Listeria with LEA. The LEA could be demonstrated only by its inoculation into susceptible sheep simultaneously with *L. monocytogenes*.

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Phenothiazine Treatment of Fattening Lambs in Drylot

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PHENOTHIAZINE has been widely used as an anthelmintic for sheep, particularly in humid or irrigated areas where opportunities for parasitism are greatest. Numerous experiments on its use in such areas have been described in the literature.

Habermann and Harwood[1] in 1939 indicated that 25 Gm. of unconditioned crude phenothiazine was effective medication against Oesophagostomum, Bunostomum, Chabertia, Haemonchus, and Ostertagia spp. occurring in sheep. Swales[2] subsequently reported in 1940, that 12.5-Gm. tablets containing 80 per cent commercial phenothiazine were apparently effective medication for sheep against Oesophagostomum, Haemonchus, Ostertagia, Trichostrongylus, and Cooperia spp. Almost simultaneously Singer and Baker[3] reported that phenothiazine seemed particularly effective for the removal of *Oesophagostomum columbianum*, *Ostertagia ostertagi*, and *Cooperia curticei*; somewhat less effective against *Nematodirus filicollis* and *Haemonchus contortus*, and ineffective for Trichostrongylus spp., Strongyloids spp., and *Trichuris ovis*. Several groups of investigators have demonstrated phenothiazine to be effective against many of the nematode parasites of sheep as measured by the criterion of fecal egg counts.[4-6]

Certain controversial results reported may be related to differences in degree of parasitic infection, or to variations in the dosage levels of phenothiazine employed. Singer and Baker[3] noted that in heavily infected sheep the average reduction in fecal egg count was 66 per cent, while in light infections the average reduction approximated 92 per cent. In English field trials, Robertson[7] found administration of three 10-Gr. phenothiazine tablets to "moderately-infested" pasture fed lambs to be ineffective in increasing weight gains or in reducing the number of worms in the abomasum, as determined postmortem. However, phenothiazine given in liquid form at the rate of 0.25 Gm. per pound of body weight to ewes caused an average increase of 7.5 lb. per head over controls during a two-month period, and a marked reduction in the number of worms. Britton and Miller[8] found that a single drench of 15 Gm. of phenothiazine was not effective in completely removing worms from lambs on irrigated Ladino clover pasture over a two-month period. However, a mixture of one part phenothiazine to ten parts of salt kept before the lambs continuously did result in increased weight gains and lowered the worm counts in the stomachs.

Fewer reports exist concerning the efficacy of phenothiazine treatment of fattening lambs under drylot conditions. Kerruish[9] reported that feeder lambs given 10 Gm. of phenothiazine in tablet form averaged 21 lb. per head gain during an 11-week feeding period, as compared with 15 lb., 7 oz., for untreated controls.

In some parts of Oregon, many of the feeder lambs come from irrigated pasture areas or other places conducive to the build-up of parasitic infection. Parasites specifically identified as troublesome in such areas include: *Haemonchus contortus*, Ostertagia spp., Trichostrongylus spp., Nematodirus spp., *Chabertia ovina*, Oesophagostomum spp., *Trichuris ovis* and Cooperia spp.[10] It was decided to test the efficiency of phenothiazine treatment of feeder lambs brought in from pasture land grazed by sheep for many years and on which no definite parasite control program had been in effect. In addition to estimates of parasite load in the abomasum made postmortem, items of commercial interest to the sheep feeder, such as weight gains, carcass grade, and dressing percentage, were calculated and compared against untreated lambs. The lambs did not give gross evidence of parasitism at the start of the experiment.

MATERIALS AND METHODS

Forty crossbred feeder lambs were randomly grouped into four lots of 10 lambs each and were housed in identical pens. Rations fed were composed of grass silage made with various additives, plus a grain mixture of equal parts of whole barley and oats. The grain was fed at a rate of 1 lb. per head daily and the silage was offered *ad libitum*. Half of the lambs in each lot chosen at random were drenched twice with 25 Gm. of phenothiazine suspension, N. F., the first time at the beginning of the experiment; the second time, two weeks later. Thus, from the four lots of lambs, there was a total of 20 treated and 20 untreated controls. All animals were weighed individually each

TABLE 1—Comparison of Growth in Treated and Untreated Lambs

Item	Phenothiazine treated	Untreated
Final group weight	1820.0 lb.	1513.0 lb.
Initial group weight	1547.0 lb.	1454.0 lb.
Total group gain	273.0 lb.	59.0 lb.
Average lamb gain	13.6 lb.	3.1 lb.
Death losses	0	1

Technical paper 932 from the Oregon Agricultural Experiment Station, Corvallis, Department of Veterinary Medicine (Allen) and the Department of Animal Husbandry (Oldfield).

The phenothiazine used in this experiment was furnished by the E. I. du Pont de Nemours Co., Wilmington, Del. The authors acknowledge with thanks the cooperation of Paul M. Rutland in care of the experimental animals and of Dr. Lyle D. Calvin and Allen F. Anglemier in the statistical treatment of data.

week. At the conclusion of the experiment, after 70 days in the feedlot, the animals were slaughtered as a group and carcass data were obtained. At the time of slaughter, blood samples were collected over sodium citrate and hemoglobin determinations made using the Spencer hemoglobinometer (American Optical Co.). The abomasums of all lambs were removed from the viscera at the abattoir and taken to the laboratory where the nematodes were estimated by the dilution method recommended by Price.[12] The abomasums were carefully washed, their contents were made up to 1,000 ml. with distilled water and vigorously shaken in a jar; a 20-ml. sample was removed for counting on a ruled Petri dish. This procedure was repeated five times, and the results were averaged.

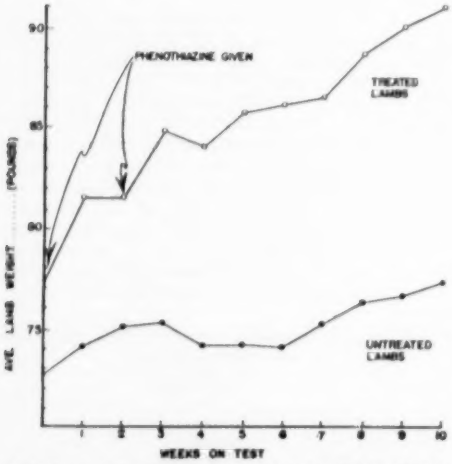
TABLE 2—Relationship Between Parasite Loads* and Certain Carcass Characteristics

Treated Group				Untreated Group			
Carcass grade	No. of lambs	Dressing (%)	Parasite load	Carcass grade	No. of lambs	Dressing (%)	Parasite load
Choice	7	52	359	Choice	2	50	1,390
Good	13	51	415	Good	11	49	1,011
Utility	0	....	....	Utility	6	48	2,122

* Average number of nematode parasites per abomasum.

EXPERIMENTAL RESULTS

A general summary of the animal growth during the experiment is presented in table 1. The treated lambs appeared in better condition throughout the experiment, and this difference is reflected in the weight gains. Hemoglobin determinations at slaughter revealed levels of 11.5 Gm. per 100 cc. in the treated animals, as compared with 10.8 Gm. in the untreated lambs. The difference in gains between the animals in the treated and untreated groups proved statistically significant ($P < .01$). Since there were appreciable weight differences at the start of the experiment, analysis of covariance was carried out using initial weight as the covariant to allow for variation of starting weights of the animals. The weight gains were not sufficiently related to initial weight to change the conclusions. Differences in hemoglobin levels were not statistically significant ($P > .05$).



Graph 1—Average weights of the lambs in the phenothiazine-treated and untreated groups during the experiment.

There appeared to be an immediate response to phenothiazine administration in terms of increased weight gains among the treated lambs. Following the second dosage, the weight gains of the treated lambs were consistently greater than those of the untreated controls for the duration of the experiment (graph 1).

Perhaps even more striking are the relationships between the carcass quality, as indicated by government grade and dressing percentage, and the postmortem worm counts made on the abomasum contents. Parasite loads were considerably lower in the treated group, and the carcass grades were consequently higher (table 2). The difference in parasite load between the treated and nontreated animals proved statistically significant ($P < .01$).

The species of nematodes encountered in the abomasums of lambs of this experiment were: *Ostertagia circumcincta*, *Haemonchus contortus*, and *Trichostrongylus axei*. Although complete differential counts were not made, Ostertagia spp. were observed to be by far the most prevalent in the abomasum. *Haemonchus contortus* was found in only 5 of the 40 lambs. In addition, intestinal worms were identified by Baker[11] as follows: *Trichostrongylus colubriformis*, *T. vitrinus*, *Cooperia curticei*, and *Bunostomum trigonocephalus*.

SUMMARY

An experiment is reported wherein ad-

ministration of phenothiazine suspension, N. F., in two 25-Gm. doses gave beneficial responses in fattening lambs under drylot conditions in terms of increased weight gains and consequently improved carcass quality. It would appear that phenothiazine is most effective in reducing moderately heavy infections (1,000 to 2,000 worms per lamb) to a point (500 or less) where the infection is not so serious in its effect upon the lamb as to affect weight gains and carcass characteristics significantly.

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An Observation on the Relationship of Cobalt Deficiency to Internal Parasites of Sheep

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In 1949, a project to determine if a relationship exists between parasitism and cobalt deficiency in ruminants was begun at this station. The need for cobalt as a dietary factor in sheep has been known for many years.

Likewise, its importance as an adjunct to anthelmintics was indicated by Winchester and Herrick [12] in 1946 and by Pope, Phillips, and Bohsted [4] in 1947. Keener,[3] in his experimental results and discussion, indicated that his results were somewhat complicated by parasitic infections in his experimental animals and that the lambs fed cobalt carried heavier worm burdens than the deficient lambs but made better gains in weight. In 1948, Weir *et al.*[11] concluded that basal rations with a mineral supplement were beneficial to lambs given an initial dose of 4,000 infective *Haemonchus contortus* larvae, followed by an additional dose of 15,000 infective larvae. Lambs receiving 45,000 larvae, with and without a smaller initial dose of 4,000 larvae, while on a low protein ration, died within six weeks. Rations containing the higher level of protein apparently increased the resistance of the animal to parasitic infection yet more worms were found in the abomasum of lambs, which received minerals, than in lambs which did not receive minerals. Whether the effect of the minerals was due to a specific element in the mineralized salt or in the bone meal was not determined.

In 1954, Richard *et al.*[5] in their study "Effect of Phlebotomy versus Stomach Worms" concluded that, "Increasing the protein with or without minerals appeared to be of little value in increasing the resistance of lambs to stomach worm infection." However, these workers [6] later reported that the feeding of cobalt and steamed bone meal were factors in increasing resistance to stomach worm infection.

"The Effect of Various Minerals on the Growth of *Haemonchus contortus* Larvae" was reported by Shumard and Herrick,[9] who stated that "Fecal material from lambs receiving iodized salt, cobaltized salt, salt containing manganese, and trace mineralized salt (Co, Mn, Fe, Cu) was used as culture medium. The length of the third stage infective larvae was used as an assay. The length of the larvae varied significantly according to the nutrition of the lamb in which the parents of the larvae resided; whereas the variation in culture media had little effect on the difference in length of the larvae." Larvae from eggs from lambs receiving cobalt or trace mineralized salt were of a significantly greater length than larvae from eggs from lambs receiving manganese or iodized salt.

In the present study, only the work pursued in 1953 is reported since cobalt deficiency was not attained in the previous trials. The lambs utilized in all trials were produced from the same ewes which were maintained under identical conditions.

Experimental Procedure

Sixteen lambs, born between Jan. 14 and Feb. 3, 1953, were removed from their mothers within one week after birth and placed in individual pens under infrared lamps in a tightly constructed barn. For three months all were weighed weekly and fed cow's milk *ad libitum*. They were then transferred to aluminum-lined cages and gradually placed on a cobalt-deficient diet consisting of cobalt-deficient timothy hay, yellow corn, and cob meal to which was added 1.5 lb. of urea, 1.0 lb. of a 2:1 mixture of sodium chloride and tricalcium phosphate, and 20.0 Gm. of halibut liver oil per 100 lb. This was designated as the basal ration. The hay was core-tested and analyzed for cobalt according to the method of Beeson [1] as modified by Price *et al.*[7] Bales showing more than 0.05 p.p.m. of cobalt were discarded.

Blood from the jugular vein was taken weekly and examined for hemoglobin content and packed cell volume. At the same time, the animals were weighed and fecal samples were collected for determination of egg counts. On April 4, the lambs were separated into groups of fours as nearly similar as possible with regard to packed blood cell volume, hemoglobin, and weight.

By June 4, 1953, the packed cell volume and grams of hemoglobin had begun to drop and weight gains decreased almost to zero, indicating that a cobalt-deficient state was being attained. Special feeding began on June 10 (table 1).

Group 1 received the basal ration plus 1 mg. of cobalt twice weekly for the first two weeks and then 1 mg. of cobalt each week until the experiment terminated.

Group 2 was fed the same basal ration and cobalt as group 1 except that on July 2 each lamb was given, orally, 70,000 larvae, comprising approximately 90 per cent *H. contortus*, 5 per cent *Ostertagia circumcinta*, and 5 per cent *Trichostrongylus axei*. The larvae were cultured from pooled samples of feces obtained simultaneously

From the Virginia Agricultural Experiment Station, Blacksburg.

TABLE 1—Body Weight, Hemoglobin, and Hematocrit Readings in Lambs as Influenced by Dietary Cobalt in the Infected and Uninfected Animals

Group	Animal No.	Survival* period after infection (weeks)	Weight (lb.)			Hemoglobin (Gm./100 cc.)			Hematocrit Readings (%)		
			June 10	Final	Gain or loss	June 10	Final	Gain or loss	June 10	Final	Gain or loss
1											
Basal	163[L1]	....									
ration	69[L2]	....	27.00	25.00	−2.00	7.75	7.75		18	18	
and	107	....	27.00	54.00	+27.00	8.5	12.0	+3.5	22	28	+6
cobalt	18	....	44.00	60.00	+16.00	10.6	12.0	+1.4	24	30	+6
2											
Basal ra-	96	5	52.00	56.00	+4.00	11.0	5.3	−5.7	28	8	−20
tion and	74	3	38.00	47.00	+9.00	10.0	5.3	−4.7	24	8	−16
cobalt and	168	2	35.00	42.00	+7.00	12.0	11.6	−0.4	29	31	+2
parasites	2	3	43.50	44.00	+0.50	9.3	6.2	−3.1	21	16	−5
3	143	6	39.00	31.00	−8.00	7.1	5.3	−1.8	16	4	−12
Basal	80**		32.00	35.50	+3.50	9.0	5.3	−3.7	20	12	−8
ration	287	2	24.00	22.00	−2.00	9.6	5.3	−4.3	23	4	−19
and	108	5	34.00	31.50	−2.50	10.0	5.3	−4.7	24	6	−18
parasites											
4	89[L2]	....									
Basal	40	....	42.00	36.50	−5.50	10.0	5.3	−4.7	23	12	−11
ration	88	....	46.00	32.00	−14.00	9.6	6.0	−3.6	23	13	−10
only	38	....	53.00	41.00	−12.00	11.0	5.3	−5.7	31	13	−18

Cobalt was administered beginning June 10, 1953, and parasites were administered on July 2, 1953; * nonparasitized animals were slaughtered on September 17; ** slaughtered on September 17; [L1] died 6 days before June 10; [L2] died 12 days after June 10; [L2] died 3 days before June 10.

from 2 host animals. The number of larvae administered was measured volumetrically by centrifugation.

Group 3 received the basal ration, without cobalt, plus approximately the same number of species of infective larvae obtained from the same host animals on the same date as group 2.

Group 4 received the basal ration alone.

Necropsies were performed on all animals. The number of parasites recovered from lamb 287 was counted (8,000), identified, dried, and weighed. The parasites recovered from the other animals were also dried and weighed. The difference in the weights of the worms from each animal served as the indication of the degree of parasitism.

Parasites recovered from animals in groups 2

TABLE 2—Influence of Dietary Cobalt on Parasitic Recoveries and Cobalt Concentration in Parasites, Liver, and Rumen Contents of Lambs

Lambs		Parasites recovered Av. wt. (Gm.)	Cobalt content (parts per million dry basis)		
Group and animal No.	Treatment		Liver	Rumen contents	Parasites
Group 1					
163	Basal	.....	0.212	.....	.....
69	ration		0.236	2.08	
107	and		0.280	0.63	
18	cobalt		0.334	0.88	
Av.			0.265	1.19	
Group 2					
(Infected)	Basal				
96	ration	1.0895	0.260	.....	0.789
74	and	0.6506	0.200	1.210	0.891
168	cobalt	0.6340	0.250	0.450	0.200
2		0.5778	0.192	0.024	0.899
Av.		0.7379	0.225	0.561	0.694
Group 3					
(Infected)					
143	Basal	0.1579	0.028	0.064	Total
80	ration	.....	0.086	0.026	of 3
287		0.0537	0.008	0.070	Lambs
108		0.3158	0.006	0.004	0.227
Av.		0.1758	0.032	0.041	0.075
Group 4					
89*			0.004*	0.005*	
40	Basal	.....	0.060	0.056	.....
88	ration		0.066	0.118	
38			0.050	0.016	
Av.			0.059	0.063	

* Not included in the average.

and 3 were analyzed for cobalt by the method mentioned. The samples were thoroughly washed in order to insure minimum surface contamination with cobalt. Haemonchus worms were classified, according to Roberts,[7] by the type of their linguiform process. A comparison also was made of the number of gravid *H. contortus* females found in 100 worms picked at random from infected lambs at necropsy.

RESULTS

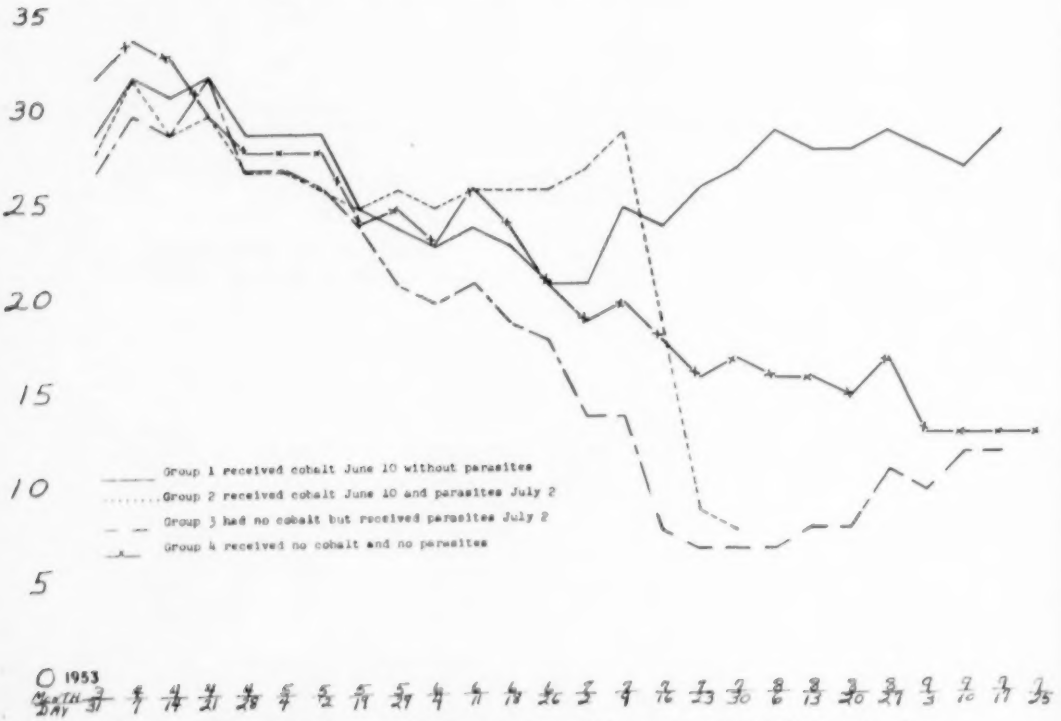
The parasitic findings were much greater in the lambs receiving cobalt than in the cobalt-deficient groups. The number of *O. circumcincta* and *T. axei* encountered comprised less than 1 per cent of the worms recovered in all of the lambs and were not considered to have any influence on the results of this study.

Hemoglobin, hematocrit, and body weight data are recorded (table 1, graphs 1, 2, 3). The parasites recovered and the cobalt concentration in liver, in rumen contents, and in parasites are also recorded (table 2). Terminal cobalt concentration in the livers indicated that the dietary treatment was effective in depleting the animals' stores of cobalt. The data on the cobalt concentration in the recovered worms are of particular interest, since no published information on this subject was found. The results strongly indicate that the concentration of cobalt in the parasites is profoundly influenced by its concentration in their environment. Furthermore the direct relationship between cobalt concentration in parasites and their ability to propagate, based on the weight of parasites recovered, is an indication that cobalt may be an element of nutritional importance to the worms.

It is of interest that the circulating hemoglobin continued to decline in heavily parasitized animals receiving cobalt, despite their apparent normal ability to store cobalt in the liver.

GROUP HISTORIES

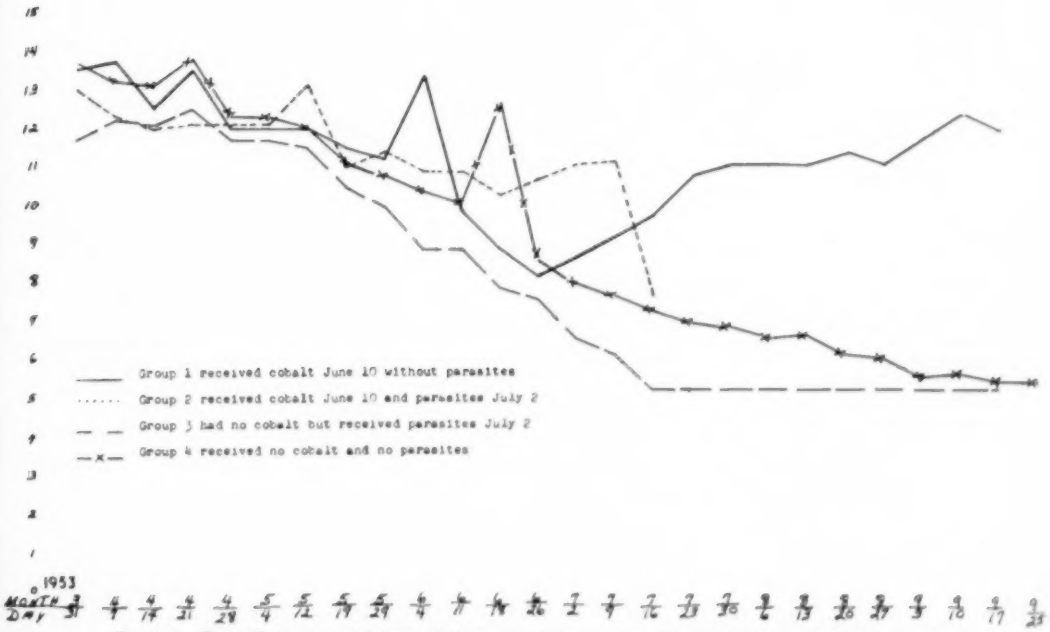
Group 1 (Uninfected, Basal Ration, Plus



Graph 1—The effect of parasitism and dietary cobalt on the packed blood cell volume (hematocrit %) of sheep.

Cobalt).—This group of 3 lambs gained the most weight despite the fact that lamb 69 died 12 days after the experimental feeding of cobalt was initiated. (Lamb 163 had died June 4.) The pathological lesions in these lambs consisted of focal areas of myocardial degeneration. The muscle fibers showed loss of striation, hyalinization of the sarcoplasm, and droplet formation; intact nuclei and sarcolemma; accumulations of histiocytes and lymphocytes in the degenerating areas and along the connective tissue septums in apparently unaffected areas; and no evidence of regeneration. The cobalt content of their livers was slightly lower than that of their group mates, 18 and 107, but the data did not suggest cobalt depletion. (Rumen sample of lamb 69 was 2.08 p.p.m.) These lambs apparently died of undetermined causes. The increase of appetites, body weights, and the maintenance of normal blood pictures combined with favorable analyses of the livers indicated that lambs 18 and 107 had responded to the weekly administration of cobalt.

Group 2 (Infected, Basal Ration, Plus Cobalt).—The infection with *H. contortus* in this group was massive, there being very few of other species of the family Trichostrongylidae involved. With the exception of lamb 168, the health of these lambs appeared to be about the same. They gained weight from the time of infection, July 2, until necropsy. A decline in hemoglobin and hematocrit readings began July 9 terminating at 6.2 or less and —10, respectively. Lamb 168 showed little deviation from the normal in any respect. This animal gained 2 lb. in the seven days following administration of infective larvae and maintained this increase in weight until its death two weeks postinfection. Since this animal died before the parasites became mature, no eggs appeared in the fecal samples and the cobalt in the parasites recovered was lower than from other animals in this group. All lambs in this group, except 96, died before egg counts became significant. Examination of the parasites in lamb 96 revealed that of 100 *H. contortus* individuals selected at random, 40 were females, all the females were gravid, and that the linguiform processes were of type 1 in 27, absent in 11 (type 2), and of abnormal types (4, 5, 6) in 2. The average

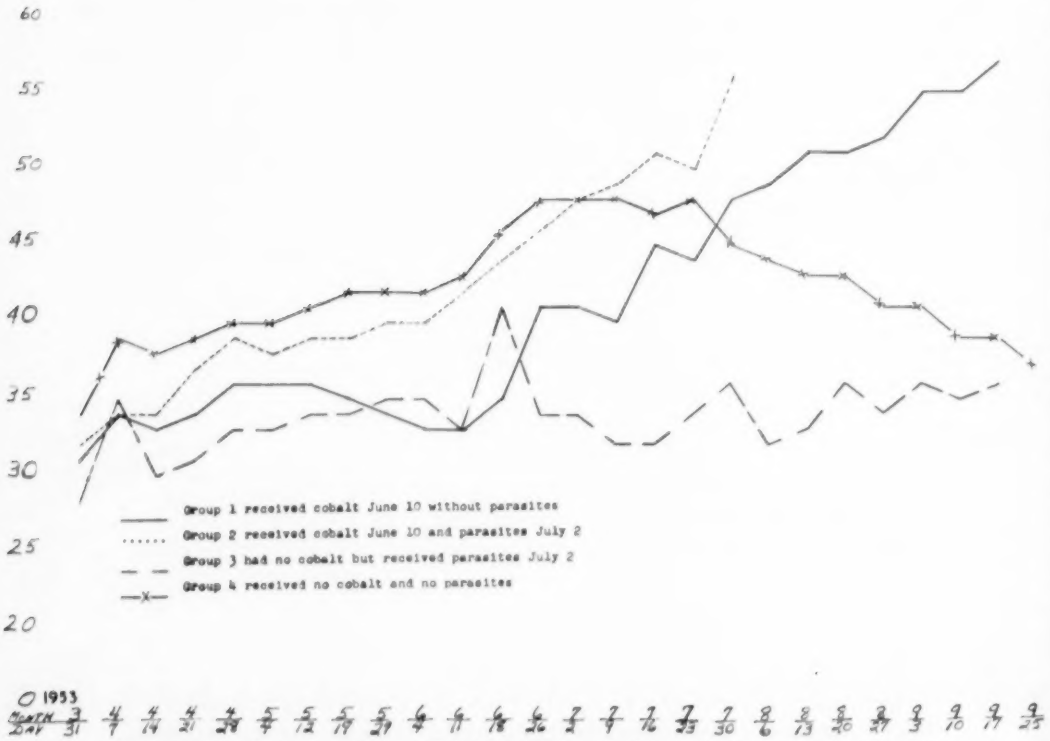

Group 1 received cobalt June 10 without parasites
Group 2 received cobalt June 10 and parasites July 2
Group 3 had no cobalt but received parasites July 2
Group 4 received no cobalt and no parasites
1953

Graph 2—The effect of parasitism and dietary cobalt on the hemoglobin content (Gm./100 ml.) of sheep blood.

length of females was 1.73 cm. The egg count, the greatest in any of the lambs, rose from 16 eggs per gram (e.p.g.) to 4,354 in seven days.

The younger parasites *(H. contortus)* in the other lambs possessed either undeveloped processes of type 1 or no process, type 2. Segmenting eggs could not be recognized. Chemical analyses of the livers of the lambs in this group yielded fairly constant values and the average was almost as high as in group 1. There was considerable variance in the cobalt concentration of the rumen contents and the average in the 3 animals available was about half that found in the animals of group 1. Considerably more parasites were recovered from the lambs in this group than in group 3 (infected, basal) and, with the exception of lamb 168, the p.p.m. of cobalt was greater in the parasites recovered from each lamb in group 2 than in the parasites recovered from any lamb in group 3.

Group 3 (Infected, Basal Ration).—There was a gradual decrease in grams of hemoglobin in all of these lambs from an average of 11.93 to less than 5.3 and the average hematocrit readings dropped from 27.0 to 6.5 per cent. Three lambs began to lose weight in June and continued until necropsy, but lamb 80 maintained its weight of 34 lb. This lamb had more cobalt in its liver than was found in the livers of the other animals. Its parasite burden was not determined because the digestive tract had been accidentally discarded. Its highest and final count of trichostrongylid-type worms (240 e.p.g. and *Strongyloides papillosus*, 408 e.p.g.) was recorded September 17. Lamb 143 yielded its final and highest egg counts of trichostrongylid-type worms (2,046 e.p.g. and *S. papillosus*, 184 e.p.g.) on August 13; lamb 287 registered 112 e.p.g. of *S. papillosus* on July 17, two days before necropsy; and lamb 108 showed 74 e.p.g. of the tri-



Graph 3—The effect of parasitism and dietary cobalt on the body weights (in pounds) of sheep.

chostrongylid type and 1,056 e.p.g. of *S. papillosus* four days before necropsy.

Of 100 Haemonchus taken from lamb 108 five weeks postinfection, 51 per cent of the females were not gravid and 20 per cent possessed an abnormal linguiform process. Of the 100 Haemonchus taken from lamb 143 six weeks postinfection, $12\frac{1}{2}$ per cent of the females were not gravid and 15 per cent possessed an abnormal linguiform process. The average lengths of the female *H. contortus* in lambs 108 and 143 (6 weeks old) were 1.41 cm. and 2.00 cm., respectively.

Group 4 (Uninfected, Basal Ration).—The biological record of animal 89, which died June 7 three days before cobalt feeding was initiated, showed a loss of 5 lb. in weight in less than three weeks, as well as a drop of 2.1 Gm. in hemoglobin, and four points in hematocrit readings. Probably the most significant findings were only 0.004 p.p.m. of cobalt in its liver and 0.005 p.p.m. in the rumen contents. Cobalt deficiency was manifested in this group in the later weeks by the loss in weight, precipitous drop in hemoglobin and in hematocrit readings, and an average cobalt level of 0.059 p.p.m. in the liver.

SUMMARY

Sixteen lambs were weaned and placed on a cobalt-deficient diet within one week after birth. Upon the manifestation of cobalt-deficient symptoms they were separated into physiologically similar groups of 4 animals each for experimental feeding and parasite infection studies.

When compared at necropsy to lambs maintained on the basal ration only, those which received cobalt also had more *Haemonchus contortus;* more of the female of these parasites were gravid; at the same postinfection interval, a higher percentage of the females had linguiform processes (types 1 and 2); and the average length of these females was greater.

More cobalt was found in the worms recovered from the cobalt-fed lambs than from the lambs fed the basal ration only.

Based on these results, the ability of *H. contortus* larvae to establish themselves within the host animal appears to be dependent upon the presence of cobalt in the diet of the host animal.

Of the animals that died after receiving infective larvae, those maintained on the basal ration plus cobalt succumbed quicker but made better weight gains than those maintained on the basal ration only

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Effects of Trace Minerals, Dicalcium Phosphate, Phenothiazine, and Combinations of These on the Resistance of Lambs to *Haemonchus Contortus* and Other Nematodes

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EXPERIMENTS conducted in drylot with single massive infection of *Haemonchus contortus*[7] indicated that addition of trace-mineralized salt and steamed bone meal to the diet increased the resistance of lambs to the effects of this parasite. It was also observed that when lambs infected with this nematode were fed cobalt, in trace-mineralized salt or "cobaltized" salt, the worms matured more rapidly and their egg production was greatly increased. It seemed probable, therefore, that the increased resistance to the effects of initial infections of *H. contortus*, in lambs on pasture, would be offset by the stimulatory effect on the development and fecundity of the parasites if the lambs were fed trace-mineralized salt and steamed bone meal.

Many investigators, among them Shorb and Habermann,[8] Britton *et al.*,[2] Andrews *et al.*,[1] Cauthen,[3,4] and Mayhew[5,6] have found that phenothiazine, administered continuously at low levels, inhibits egg production of most parasitic nematodes and retards development of larval forms.

MATERIALS AND METHODS

Fifty-two grade ewes on rations of alfalfa hay, ground corn, and iodized salt were mated with purebred Suffolk rams. Resulting lambs were creep-fed the same rations received by the ewes. Of these lambs, the older 60 were selected for the experiment when at least 1 month old. Male lambs had been castrated and all lambs were docked. Prior to placing the animals on pasture, each ewe was drenched with 4 oz. of aqueous solution containing 1.5 oz. of phenothiazine and given a capsule of lead arsenate. All ewes and their lambs were vaccinated against enterotoxemia with *Clostridium perfringens* toxoid. The animals were allotted to six different lots so that 4 ewes and their twins and 2 ewes and their single lambs were in each lot. There were thus 10 lambs and 6 ewes per lot. A large flat pasture, not previously used for cattle or sheep, was fenced so that six nearly equal-sized lots resulted. The lots varied between 155 and 162 ft. in width and all were 375 ft. long.

Covered salt boxes were utilized. Salt rations were fed beginning the first day on pasture (table 1). Unpublished data on a study of infected lambs on pasture, which served as a pilot experiment for the one being discussed, showed that steamed bone meal mixed with the salt was probably unpalatable. Dicalcium phosphate, therefore, was substituted for the steamed bone meal.

Hot, dry weather forced the removal of the animals to a neighboring pasture after they had been on the first pasture for 21 days. The second pasture had been used the previous year for sheep and, therefore, was contaminated. The lots in this second pasture averaged 230 by 125 ft.

TABLE 1—Salt Rations Fed, Free Choice, to Ewes and Their Experimental Lambs

Lot	Ration
1	Iodized salt
2	Phenothiazine (10 %*) in iodized salt
3	Trace-mineralized salt**
4	Phenothiazine (10 %) in trace-mineralized salt
5	Phenothiazine (10 %) and dicalcium phosphate (33⅓ %) in trace-mineralized salt
6	Dicalcium phosphate (33⅓ %) in trace-mineralized salt

* All percentages based on weight; ** trace-mineralized salt consisted of the following as percentages: NaCl, 96.0; Fe, 0.30; Mn, 0.150; Co, 0.010; Cu, 0.060; I, 0.007.

All lambs were given approximately 20,000 infective *Haemonchus contortus* larvae by means of a drenching syringe three days after they had been placed on the second pasture. The ewes were allowed to run with their lambs throughout the experiment.

After the sheep had been on the second pasture for 30 days (27 days after receiving larvae) weather conditions forced their removal to the first pasture. From this time on the two pastures were alternately utilized at approximately ten-day intervals, each lot retaining its individual fenced portion of each pasture.

Hemoglobin levels, fecal egg counts, and weights

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were determined prior to administration of larvae and at weekly intervals thereafter. Hemoglobin was measured by the oxyhemoglobin method; egg counts were determined by the method of Stoll.[5]

The experiment was for 117 days or 94 days following administration of the larvae. Surviving lambs were slaughtered, the abomasums removed, and the worms counted.

Results and Discussion

The feces of all lambs were negative for nematode ova prior to the sixteenth day following administration of larvae. At this time, ova were found in the feces of the following lambs: 4 lambs in lot 1 (iodized salt); 3 lambs in lot 2 (iodized salt and phenothiazine); 10 in lot 3 (trace-mineralized salt); 10 in lot 4 (trace-mineralized salt and phenothiazine); 8 in lot 5 (trace-mineralized salt, phenothiazine, dicalcium phosphate); 9 in lot 6 (trace-mineralized salt and dicalcium phosphate). The remainder of the lambs were passing eggs in their feces by the twenty-first day following infection except for 2 lambs in lot 2 which were passing eggs by the twenty-seventh day. More lambs receiving trace-mineralized salt were passing eggs at an earlier date than lambs not receiving this addition.

Approximately 60 days following infection most of the lambs in lot 3 began scouring as did a few in the other lots. The fecal material was dark brown and fluid. A few days later, eggs of Trichostrongylus sp. were found in the feces of all lambs on the experiment. A few Nematodirus sp. eggs were detected in the feces, well distributed in all lots. These two genera could have overwintered on the second pasture or have been carried by the ewes. The time of their appearance suggests the former.

Deaths from the effects of parasitic nematodes occurred in 80 per cent of the lambs in lot 3, 20 per cent in lot 1, and 10 per cent in lot 4. One of the surviving lambs in lot 3 suffered from "stiff-lamb disease" at the start of the experiment. It was treated with several intramuscular injections of wheat germ oil. The lamb recovered from the lameness and was not noticeably affected by the parasitic infections.

The action of phenothiazine on worm egg production varied according to the mineral additions to the diet (table 2). Lot 2 lambs (phenothiazine in iodized salt) exhibited lower egg counts than lambs of lot 1 (iodized salt) in all except two instances, 27 and 34 days following infection, even though the number of Haemonchus recovered at necropsy was greater in the lot 2 lambs (table 4). Lot 3 lambs (trace-mineralized salt) showed much higher egg counts than did lambs in lot 4 (phenothiazine in trace-mineralized salt). The 2 surviving lambs of lot 3 had more worms than those of lot 4 but not in proportion to the final egg counts. Lot 5 lambs with phenothiazine produced less eggs and carried fewer worms than did lambs in lot 6, which had no phenothiazine.

All lots receiving phenothiazine exhibited much lower peaks of egg production than did lots not receiving this drug. After the initial peak of egg production, the counts from the lots receiving phenothiazine leveled off while the counts from the other lots dropped rapidly and then rose to reach secondary and tertiary peaks.

The effects of the worms on the hemo-

TABLE 2—Average Number of Eggs (in Thousands) per Gram of Feces from Experimental Lambs

Days after infect.	Lot 1†	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
21	1.26	1.09	2.16	1.86	1.34	3.94
27	1.82	2.60	2.80	2.56	1.94	3.48
34	2.86	3.08	5.24	5.35	4.04	9.32
41	6.42	3.34	13.34	7.30*	1.60	5.12
48	12.14	4.92	41.78	8.38	3.06	10.82
55	10.20*	4.90	26.67**	5.86	2.26	6.90
62	13.00	2.60	28.40*	3.67	1.36	7.42
69	12.02	2.20	20.12**	3.98	1.51	8.80
76	19.20	3.10	21.92	2.98	1.60	7.38
83	7.14	3.34	15.15*	2.58	1.94	3.42
94	9.45*	4.36	12.40**	3.5	1.98	4.14

Each * indicates death of a lamb due to parasitic nematodes.

† Lot 1, iodized salt; 2, iodized salt and phenothiazine; 3, trace-mineralized salt; 4, trace-mineralized salt and phenothiazine; 5, trace-mineralized salt, dicalcium phosphate, and phenothiazine; 6, trace-mineralized salt and dicalcium phosphate.

TABLE 3—Average Hemoglobin Concentration (Gm. per 100 ml. of Blood) in Experimental Lambs

Days after infect.	Lot 1†	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
−3	12.45	12.67	12.55	12.43	11.98	12.29
5	12.44	12.20	12.67	12.63	12.20	12.91
14	12.79	12.57	12.33	12.63	12.15	12.70
21	12.73	12.73	12.21	12.22	12.38	12.50
27	12.20	12.33	12.00	11.68	12.15	11.78
34	10.69	11.45	9.98	9.75	11.56	11.09
41	9.44	11.68	7.52	8.78*	11.96	11.06
48	9.02	11.50	5.18	9.11	11.43	10.29
55	9.95*	11.92	5.96**	9.20	11.30	10.23
62	9.55	11.81	5.27*	9.68	11.12	9.53
69	8.20	11.23	6.40**	9.00	11.00	9.41
76	7.43	10.53	4.00	8.52	10.44	8.69
83	7.76	10.72	4.68*	10.35	10.97	8.23
94	8.09*	9.54	5.09**	9.26	10.58	7.47

Each * indicates death of a lamb due to parasitic nematodes.

† Lot 1, iodized salt; 2, iodized salt and phenothiazine; 3, trace-mineralized salt; 4, trace-mineralized salt and phenothiazine; 5, trace-mineralized salt, dicalcium phosphate, and phenothiazine; 6, trace-mineralized salt and dicalcium phosphate.

globin of the lambs receiving phenothiazine were less severe (table 3) probably due in part to the prevention of succeeding massive infections.

Lambs in lot 3 expelled large numbers of worm eggs upon their pastures (table 2) while lot 6, although receiving trace-mineralized salt and exhibiting the highest initial worm egg production, did not produce large numbers of eggs. This may have been due to the added resistance afforded by the addition of dicalcium phosphate although this resistance was not complete as shown by the gradual fall of hemoglobin (table 3). Lot 1 lambs exhibited the same gradual loss of hemoglobin but had higher egg counts that showed several definite peaks. This lot had fewer Haemonchus at slaughter than did lot 6 (table 4), indicating that the latter still retained a degree of resistance to the effects of the worms.

Lambs in lot 3 were severely affected by the infections although the 2 surviving lambs did not contain large numbers of stomach worms. Necropsy of lambs that died from worm infections revealed extremely large numbers of worms but no attempt was made to count them as it was sometimes impossible to examine the carcasses for some hours following death.

TABLE 4—Average Number of Haemonchus Found in Abomasums of Experimental Lambs and Percentage of Total Weight Gain

	Lot No.					
	1†	2	3	4	5	6
No. of worms	3,246	4,949	3,517	2,943	2,196	5,454
Weight gain (%)	30	58	−40	54	56	42

† Lot 1, iodized salt; 2, iodized salt and phenothiazine; 3, trace-mineralized salt; 4, trace-mineralized salt and phenothiazine; 5, trace-mineralized salt, dicalcium phosphate, and phenothiazine; 6, trace-mineralized salt and dicalcium phosphate.

Total percentage weight gains were determined by dividing the difference between the total initial weight of each lot and the total final weight by the total initial weight (table 4). All lots receiving phenothiazine had comparable weight gains in spite of the loss of 1 lamb in lot 4. Lot 6 (trace-mineralized salt and dicalcium phosphate) gained 12 per cent more weight than lot 1 (iodized salt).

SUMMARY AND CONCLUSIONS

A study has been conducted on the resistance of lambs on pasture to the effects of *Haemonchus contortus* and other nematodes when fed additions of trace minerals, dicalcium phosphate, phenothiazine, and combinations of these. This experiment showed that, in most cases, phenothiazine reduced the worm burden of the lambs; probably not by a direct lethal effect on the adult worms but rather by inhibition of egg production and hatchability of the eggs, thus preventing massive reinfections of the lambs.

Lambs receiving trace-mineralized salt, without dicalcium phosphate or phenothiazine, were most severely affected by the nematode infections. Eighty per cent of these lambs died and they contaminated the pasture with the largest number of nematode eggs. Addition of dicalcium phosphate to the diet containing trace-mineralized salt produced an added re-

sistance. Although these lambs were more infected, they gained 12 per cent more weight than lambs receiving iodized salt.

More lambs receiving trace-mineralized salt passed worm eggs the first few weeks than did lambs not receiving this salt, regardless of further addition of phenothiazine or dicalcium phosphate.

Analyses of worm egg counts, number of worms recovered at necropsy, and hemoglobin concentrations of infected lambs seemed to support the contention that a phenothiazine, trace mineral-dicalcium phosphate combination was superior to any of these alone. This suggests that for maximum control, phenothiazine is at its best when administered with this combination of mineral supplements.

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Immunizing Activity of Alum-Precipitated Protective Antigen of *Bacillus Anthracis* in Cattle, Sheep, and Swine

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THE PROTECTIVE antigen of *Bacillus anthracis* has been produced by growth of the organism in chemically defined, nonprotein mediums.[1,2]

The antigen was present in soluble form in the culture filtrates, and could be stabilized, concentrated, and partially purified by precipitation with alum.[1,3] The immunizing activity of the antigen was demonstrated in rabbits, guinea pigs, and monkeys;[3] the immunity produced in rabbits was effective against challenge with 35 strains of *B. anthracis* of varied origin.[4]

Belton and Strange[5] confirmed the elaboration of antigen in chemically defined mediums, and developed a modified medium containing casein hydrolysate. Devlin *et al.*[6] modified the mediums of Puziss and Wright,[7] and devised methods for production of the antigen on a large scale.

The present study was undertaken to investigate the immunizing activity of alum-precipitated protective antigen in cattle, sheep, and swine, and to provide an indication of the duration of the immunity.

MATERIALS AND METHODS

Antigen Preparations.—Two preparations of alum-precipitated protective antigen were used. Lot 92 was produced by growth of the NP-A strain[7] in 528 medium.[1] Lot 39-30 was produced by growth of the RI-NP strain in the modified medium described by Devlin *et al.*[6] The sterile culture filtrates were immediately precipitated with alum, washed with saline, and suspended in saline at 1/10 the culture volume.[3] Merthiolate at a concentration of 1:10,000 was added as a preservative. The antigen preparations were tested for sterility in tryptose or nutrient broth, and in Brewer thioglycolate medium. Safety tests were performed by subcutaneous injection of 5 ml. into each of 5 guinea pigs, and 1 ml. into each of 10 Swiss mice. No evidence of the presence of viable organisms was obtained by these procedures.

Antigenic potency was estimated by immunization of rabbits with dilutions of the antigen preparations, followed by a standard challenge with 10,000 spores of the Vollum strain of *B. anthracis*.[2,4] Both lot 92 and lot 39-30 were of typical activity, and immunized rabbits against the standard challenge when a total amount of 0.1 ml. of stock antigen was injected.

Strains of Bacillus Anthracis.—The NP-A and RI-NP strains that were used for elaboration of antigen were mutants of the Vollum strain and have been described by Puziss and Wright.[7] Vollum, V770, and 1062 were virulent strains of animal origin.[4] Strain V770-2-P was recovered from the blood of heifer 2728 on the fourth day following intramuscular injection of strain V770. It was then injected into a guinea pig, and recovered from the heart blood at necropsy. This strain appeared to be more virulent for laboratory animals than the parent strain. Four or more spores injected subcutaneously into guinea pigs resulted in 100 per cent mortality in three to five days. Repeated tests indicated that the lethal dose (l.d.)$_{50}$ for rabbits was approximately 50 spores when given by the same route. This strain was used for most of the challenge experiments to be reported.

Spore suspensions were prepared by growing the strains on nutrient agar for four days, suspending and washing the growth in water, and heating at 56 C. for 30 minutes. The suspensions were stored at 4 C.; under these conditions they maintained their viability and virulence for long periods. The stock suspensions were diluted in sterile water as required for challenge of animals.

Animals.—Native Michigan cattle of mixed dairy and Hereford breeds were used. They were approximately 1½ years of age and weighed between 450 and 700 lb. The sheep were good to choice slaughter ewes of mixed breeds and were obtained from the Detroit stockyards. They originated from various parts of Michigan and were fairly uniform in age and weight. Chester White pigs that weighed between 30 and 40 lb. were obtained from nearby farms. Immediately on receipt and seven days before experimental use, each pig was immunized with anti-hog cholera serum and virus.

All animals were held under the usual farm conditions until the time of challenge. They were then moved to a screened concrete building equipped with pens and stanchions. The sewage system permitted collection of all liquid wastes in storage tanks for sterilization by steam under pressure. Solid wastes were burned in an incinerator in an adjacent building. Animals that died of infection, and those that survived and were destroyed, were necropsied and blood cultures were made. The carcasses were sterilized in a reduction tank under 60 lb. of steam pressure for six

From the Research Laboratories, Parke, Davis and Company, Detroit, Mich., and Camp Detrick, Frederick, Md.

This work was carried out at Parke, Davis and Company under a contract with Camp Detrick.

hours, and the residue was dried under vacuum and burned. Surviving animals were held for at least 21 days before being killed.

EXPERIMENTAL RESULTS

Immunization of Cattle.—Twelve animals in two groups were immunized by two 5-ml. injections of antigen four weeks apart. The injections were made subcutaneously in the upper third of the neck. The antigen was well tolerated; no local or systemic reactions were noted. The first group of 8 animals received antigen lot 92. The difficulty and consequent delay in developing a satisfactory method of challenge made it necessary to immunize an additional group of 4 animals, so that challenge could be carried out one month after immunization. Antigen lot 39–30 was used for immunization of the latter animals.

Assessment of the immunizing activity of the antigen required a standardized procedure of challenge that would produce severe and preferably fatal infection in normal cattle. Attempts were made to produce fatal infection by administration of various strains by several routes (table 1). Although infections that varied from slight to severe were produced by parenteral challenge in most cases, all of the animals survived. The failure to produce fatal infection by parenteral routes is in agreement with most previous observations.[8, 9]

It was suggested from the Microbiological Research Department, Porton, England, that fatal infection of cattle could be obtained by administration of spores by the oral route. This method of challenge was investigated by feeding a pellet of food containing the spore suspension. The pellets were prepared by boring an 8-mm. hole in commercially available cylindrical pellets of cattle feed. The spore suspension was placed in the cavity and the opening was closed with a small cork. In almost all cases, the animals took the pellets voluntarily and chewed them immediately. When the pellets were dipped in saturated sugar solution or in black strap molasses, they were accepted and masticated more readily. Spores of the V770-2-P strain, which had been recovered from the blood of a heifer following intramuscular challenge, were used in these experiments (table 2).

In the first experiment, 4 animals were given 10^9 spores. Three of the animals died in less than three days; the fourth gave no evidence of infection but died promptly when rechallenged with the same dose. One animal fed 10^9 spores of the Vollum strain had a slight febrile reaction but recovered. There was no evidence of infection when the latter animal was rechallenged with 10^9 spores of V770-2-P.

Further titrations of the V770-2-P suspensions by the oral route were performed (table 2). Positive blood cultures were obtained from all animals 24 hours or more before death. Animal 362, which survived infection, also yielded positive cultures for several days. Although the number of animals was not sufficient to establish an $l.d._{50}$, it appeared that challenge with 10^7 spores was less severe than challenge with 10^9 spores, because the intervals between challenge and death were longer, and 1 infected animal survived. Accordingly, 1.5

TABLE 1—Challenge of Normal Cattle by Various Routes

Animal	No. of spores and strain	Route of challenge	Results*
2745	5×10^5 Vollum	Intracutaneous	Slight local reaction; temp. 105 F. on day 4; normal thereafter; rechallenged on day 14.
2745	3×10^8 Vollum†	Conjunctival	No reaction in 21 days.
2715	1.5×10^6 Vollum	Subcutaneous	Local reaction evident on day 4; elevated temperature day 4 through 7; recovered.
2746	5×10^6 Vollum	Subcutaneous	Local reaction; elevated temperature and anorexia day 3 through 7; recovered.
2763	6×10^8 Vollum	Subcutaneous	Elevated temperature and anorexia day 3 through 8; 107 F. on day 3; blood in feces day 13 through 15; recovered.
2712	3×10^8 Vollum	Intravenous	Local and submaxillary edema day 3 through 18; elevated temperature day 2 through 6; recovered.
2776	6×10^8 Vollum	Oral in gelatin capsule	Slightly elevated temperature day 5 through 9; recovered.
2728	6×10^8 V770	Intramuscular	Elevated temperature, anorexia, diarrhea day 2 through 6; positive blood culture on day 4; recovered.

* Reactions are recorded in terms of days after challenge. Temperatures in excess of 103 F. were regarded as elevated; † rechallenge.

TABLE 2—Virulence of V770-2-P for Normal Cattle by the Oral Route

Animal	Spores fed	Blood culture	Results *
582	10^9	Positive at 40 hours	Died 64 hours after challenge.
2	10^9	Positive at 40 hours	Died 64 hours after challenge.
569066	10^9	Positive at 45 hours	Died 68 hours after challenge.
569067	10^9	Negative	No reaction; rechallenged 7 days after initial challenge.
	10^9†	Positive at 46 hours	Died 67 hours after challenge.
25	10^8	Positive at 72 hours	Died 90 hours after challenge.
26	10^8	Positive at 96 hours	Died 149 hours after challenge.
27	10^9	Negative	Did not chew pellet; rechallenged 10 days after initial challenge.
	10^9†	Positive at 48 hours	Died 68 hours after challenge.
4756	10^7	Positive at 96 hours	Died 125 hours after challenge.
362	10^7	Positive 6, 7, 8 days	Elevated temperature day 2 through 8; survived.
268947	10^7	Positive 5, 6, 7 days	Died 165 hours after challenge.

* Necropsy revealed typical anthrax in all animals that died; † rechallenge.

x 10^8 spores was selected as the standard challenge dose, with the expectation that it would represent a severe test of the resistance of the immunized animals and would prove fatal to most of the controls. Although an occasional control gave no reaction to this or a larger dose, most of these animals died promptly on rechallenge, possibly because they had not chewed or swallowed the original pellet in the normal manner.

Five months after immunization and before the oral challenge method was developed, 3 immunized animals of the first group and 3 normal controls were challenged parenterally with large numbers of spores. The infections appeared to be appreciably more severe in the control animals, but no satisfactory objective evidence of immunity could be obtained by these challenge procedures.

The 9 remaining immunized animals and 4 normal controls were challenged by the oral route (table 3). In the initial experiment, 2 animals immunized eight months previously and 1 control were challenged with 10^9 spores. In the second experiment, 4 animals immunized one month previously, 3 animals immunized 12 months previously, and 3 controls were challenged with 1.5×10^8 spores. In this experiment, 1 of the control animals survived the initial challenge, and accordingly seven days after the initial challenge this animal and the animals that had been immunized one month previously were rechallenged with the same dose. Daily blood cultures were made from all animals until they died or were destroyed. In all those which succumbed, *B. anthracis* was found 24 hours or more before death. Blood cultures from survivors were sterile except from 2717 which was positive on the fourth day only. Evidently the animals were solidly immune one month after immunization. After eight and 12 months the immunity had declined, but the results suggest that the animals

TABLE 3—Oral Challenge of Cattle at Various Intervals After Immunization

Animal	Months after immunization	Spores per animal *	Results
2758	8	10^9	Died of anthrax after 148 hours.
2717	8	10^9	Elevated temperature and anorexia day 3 through 5; survived.
Control T569063	....	10^9	Died of anthrax after 67 hours.
21	1	1.5×10^8†	No reaction.
22	1	1.5×10^8†	No reaction.
23	1	1.5×10^8†	No reaction.
24	1	1.5×10^8†	No reaction.
2734	12	1.5×10^8	Died of anthrax after 148 hours.
2757	12	1.5×10^8	Elevated temperature day 3 through 5; survived.
2716	12	1.5×10^8	Elevated temperature day 3 and 4; diarrhea and anorexia day 4 through 7; survived.
Control 8-9	....	1.5×10^8	Died of anthrax 80 hours after challenge.
Control 8-10	....	1.5×10^8	Died of anthrax 102 hours after challenge.
Control 29	....	1.5×10^8†	No reaction to first challenge; died of anthrax 91 hours after rechallenge.

* Strain V770-2-P; † rechallenged with the same dose on the seventh day after the initial challenge.

were significantly more resistant than the controls.

Immunization of Sheep.—Twenty-five animals were immunized by subcutaneous injection of two 5-ml. doses of antigen 39-30 at an interval of four weeks. The injections were made into the medial surface of the right flank, an area devoid of wool. No local or systemic reactions were noted.

No difficulty was encountered in determination of a fatal challenge dose for sheep. In agreement with previous reports, these animals were readily infected by subcutaneous challenge and died promptly with generalized anthrax. Results of titration of the V770-2-P strain in sheep are presented (table 4). The $l.d._{50}$ was estimated to be of the order of 100 spores, and a standard dose of 20,000 spores was selected for challenge of immunized and control sheep.

Groups of immunized animals were challenged 1, 3, 6, 7, and 8 months after the second injection of antigen (table 5). Normal control animals were challenged with each group. Surviving animals were observed for at least 21 days before being destroyed and necropsied. No abnormalities referable to the experimental procedures were detected at necropsy of the survivors.

Blood cultures were made from all surviving animals three, seven, 11, and 21 days after challenge in the 1-month group, and three, nine, 15, and 21 days after challenge in the other animals. In addition, daily blood cultures were made during febrile periods. Positive cultures were obtained from 2 animals, in each case 12 to 16 hours before death.

Immunization of Swine.—Fifteen animals were given two 5-ml. doses of antigen 39-30 at an interval of four weeks. The injections were made intramuscularly in the left hindleg; no local or systemic reactions were observed, and the rate of growth of the immunized animals appeared normal.

Attempts to produce fatal infection in 15 normal swine with strain V770-2-P were unsuccessful. Subcutaneous injection of 1.5×10^8 spores produced a slight local reaction but no other symptoms. Feeding of 10^8 spores or intranasal or intratrachial instillation of 7.5×10^7 to 3×10^8 spores produced no evidence of infection. Trombitas[10] reported that all of 14 swine died of anthrax after feeding on a cow dead of anthrax. Accordingly 2 swine were each fed a carcass of a guinea pig recently dead of anthrax. One guinea pig had been infected with strain V770-2-P, and the other had received strain 1062. The latter strain had been isolated from a natural infection in swine.[4] The carcasses contained innumerable anthrax bacilli and were completely consumed by the animals. Both swine showed a febrile response of 48 hours' duration following challenge but survived.

The feeding of infected guinea pigs produced the most severe symptoms of any of the methods that were investigated. Accordingly at two and at five months after immunization, 4 immunized animals and 4 controls were challenged by feeding carcasses of 400- to 700-Gm. guinea pigs that had died following injection of 10,000 spores of strain V770-2-P (table 6). Infec-

TABLE 4 — Virulence of V770-2-P for Normal Sheep

Spores injected*	Animals dead† / animals challenged	Interval between challenge and death (days)
500	3/3	4, 5, 6
250	2/3	4, 5
50	1/3	6

*Spores were diluted in water and injected subcutaneously in a volume of 1 ml.; †necropsy revealed typical anthrax in all animals that died.

TABLE 5—Challenge of Sheep at Various Intervals After Immunization

Interval between immunization and challenge (months)	Survival ratio*		Remarks
	Control animals	Immunized animals	
1	0/3 (3, 3, 3)	3/5 (6, 7)	No reaction to challenge in survivors.
3	0/3 (2, 3, 4†)	5/5	No reaction to challenge.
6	0/3 (3, 4, 4)	2/5 (2, 15, 20†)	No reaction to challenge in survivors.
7	0/3 (2, 3, 3)	1/5 (4, 5, 8, 11)	Elevated temperature for 2 days in survivors.
8	0/2 (2, 4)	3/4 (7)	Elevated temperature for 1 to 3 days in survivors.

*Each animal was injected subcutaneously with 20,000 spores of strain V770-2-P. Survival ratios are recorded as the number of animals surviving divided by the number challenged. Numbers in parentheses refer to the days after challenge on which deaths occurred. Septicemia and gross pathology typical of anthrax were present in all animals that died.

†Positive blood culture obtained 12 to 16 hours before death.

TABLE 6—Challenge of Immunized and Normal Swine by Feeding Carcasses of Guinea Pigs Dead of Anthrax

Animal	Results
GROUP 1—CHALLENGED 2 MONTHS AFTER SECOND IMMUNIZING INJECTION	
2373*	No reaction.
2381*	No reaction.
2392	106 F. on day 1 after challenge; normal thereafter; pharyngeal swelling for 2 days.
2396	Elevated temperature on days 1 and 2 after challenge with anorexia; slight pharyngeal swelling.
Control 1*	Elevated temperature days 3 through 5 with anorexia; marked pharyngeal swelling for 3 days.
Control 2	Elevated temperature days 1 through 5 with anorexia and pharyngeal swelling.
Control 3	Elevated temperature days 1 through 7 with anorexia and pharyngeal swelling.
Control 4*	Elevated temperature on day 2 only; slight anorexia and pharyngeal swelling for 7 days.
GROUP 2—CHALLENGED 5 MONTHS AFTER SECOND IMMUNIZING INJECTION	
2390	Elevated temperature days 1 through 4.
2391**	Elevated temperature days 3 and 4 with slight anorexia.
2394	No reaction except slight anorexia on day 3 and slight pharyngeal swelling.
2395**	Elevated temperature days 3 and 4 with slight anorexia.
Control 1**	Elevated temperature days 2 and 3.
Control 2	Elevated temperature days 1 through 5 with anorexia; slight pharyngeal swelling.
Control 3	Elevated temperature days 2 through 5 with slight anorexia.
Control 4**	Elevated temperature days 1 through 5 with slight anorexia.

* These swine were fed a guinea pig carcass on each of 2 successive days.

** These swine were fed a guinea pig carcass that had been kept at about 10 C. for 24 hours.

tions of varying severity were produced in the controls. The immunized animals that were challenged two months after immunization appeared to be partially protected, in that 2 animals showed no evidence of infection; in the other animals, the infections were apparently milder than in the controls. Five months after immunization the resistance had apparently decreased, and little objective evidence of immunity was obtained.

DISCUSSION

The experiments clearly indicate that the alum-precipitated protective antigen produced effective immunity in cattle and in sheep. The 4 cattle that were challenged one month after immunization were evidently completely resistant to a challenge procedure that proved fatal to the 3 controls. During titration of the challenge suspension, similar or smaller doses proved fatal to 4 of 6 normal animals and produced a severe infection in the fifth. Eight and 12 months after immunization, the immunity had evidently decreased. All of the 5 immunized animals became infected and 2 died. It seems probable that significant immunity remained, however, because the deaths were delayed, as compared with controls, and because 3 of the 5 animals survived infection. Recovery occurred in only one instance among normal animals infected by the oral route.

Significant immunity was present in sheep one month after immunization, but better protection was obtained after three months. The immunity had decreased six, seven, and eight months after immunization, but was sufficient to prevent death in 6 of the 14 animals, and to delay it in most of the others. The standard challenge dose represented approximately 200 $l.d._{50}$ and was rapidly fatal to all of 17 control animals.

The challenge procedures for cattle and sheep were adjusted to provide a severe test of the resistance of the immunized animals. The V770-2-P strain that was used was extremely virulent for laboratory animals, and Auerbach and Wright [4] reported that this strain produced fatal infections in guinea pigs that had been immunized by procedures that protected them against many virulent strains. Accordingly, it is difficult to estimate the duration of effective immunity under field conditions on the basis of the present experiments. It would seem probable that the infective doses that would be encountered under natural conditions would be less severe than those employed in the present experiments.

Most previous assessments of anthrax immunization procedures have been carried out in sheep [11] * because of the failure of parenteral methods of challenge to produce fatal infection in normal cattle. The oral challenge procedure should prove useful for future evaluation of immunization procedures in these animals. It is clear that oral administration of large numbers of highly virulent spores produces fatal infection in a large proportion of normal cattle. The relative importance of the virulence of the strain and the route of infection can not be determined on the basis of the present data. The results suggest that

* Since completion of these experiments Boor and Treselt [12] reported protection of sheep by immunization with repeated doses of an aqueous solution of cell-free antigen or by a single dose of the antigen adsorbed on colloidal aluminum hydroxide.

both a strain of maximum virulence and the oral route of infection are necessary for fatal infection.

The present immunization studies must be regarded as exploratory. The doses of antigen and schedules of immunization were arbitrarily selected, and presumably can be modified for greater effectiveness. The slow development of maximum immunity in sheep suggests that an excessive dose of antigen may have been used in these animals. The protective activity of the crude filtrate can doubtless be increased by further investigation, and the possibility of a more effective adjuvant than alum should also be explored. It is evident from the present study that cattle and sheep can be immunized against anthrax with a sterile, nontoxic antigen preparation. Additional studies are necessary to improve and simplify the methods and to determine their practical value under field conditions.

SUMMARY

The immunizing activity of the protective antigen of *Bacillus anthracis* was investigated in cattle, sheep, and swine. The animals received two injections of the alum-precipitated antigen four weeks apart and were challenged with a highly virulent strain of *B. anthracis* after various intervals. Cattle were challenged by oral administration of 150 million spores, which proved fatal to most of the control animals. Sheep were challenged subcutaneously with 20,000 spores; this dose represented approximately 200 lethal dose$_{50}$. Fatal infections were not obtained in normal swine, although moderately severe infections were obtained by feeding carcasses of guinea pigs dead of anthrax. Solid immunity was present in cattle one month after immunization; the immunity had declined but appeared significant after eight and 12 months. Sheep were solidly immune three months after challenge; less than complete survival was obtained one, six, seven, and eight months after immunization. Estimation of the immunity of swine was difficult because fatal infection could not be produced in normal animals. Results that suggested increased resistance were obtained two months after immunization.

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Serological Differentiation of the Equine Abortion Virus from the Human and Swine Influenza, Mumps, and Newcastle Disease Viruses

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EQUINE virus abortion was characterized as a specific entity by Dimock and coworkers.[4-7] Their investigations were restricted principally to studies on aborted fetuses, transmission experiments, and inoculation of laboratory animals. Effects of the abortion virus on the mare and other horses were not studied.

During investigation of the disease in Hungary, Manninger and Csontos[25] and Manninger[26] observed febrile reactions and signs of mild respiratory catarrh in mares inoculated with the abortion virus. These mares were immune to subsequent inoculation with blood from horses believed to have equine influenza, and horses which had recovered from a febrile disease diagnosed as equine influenza were immune to the equine abortion virus. They concluded that virus abortion was a manifestation of infection of pregnant mares by the equine influenza virus.

In an investigation of the relation of equine abortion virus and two viral isolates designated as equine influenza virus, Doll and Kintner[8] found reciprocal complement fixation between the abortion virus and the influenza viruses and their respective antiserums. When inoculated into equine fetuses *in utero*, the equine influenza viruses caused abortion and the aborted fetuses had lesions typical of equine virus abortion. In a cross-protection test, infection by the abortion virus immunized young horses against subsequent inoculation with the equine influenza viruses.

Doll *et al.*[9] found that young horses inoculated with the equine abortion virus developed a fever, leukopenia, and signs of mild respiratory catarrh. The abortion virus infection closely resembled the uncomplicated equine influenza reported by Jones *et al.*[10, 29] and Maurer and Jones.[28]

The equine abortion virus and two viral isolates that were designated as equine influenza virus (Army 183[28] and Grayson I[9, 29]) were adapted to hamsters and subsequently were cultivated in chicken embryos by Doll *et al.*[10-14] In these hosts the abortion and influenza viruses produced identical lesions, and infectivity of either virus was neutralized completely by antiserums for the other.

The identity of the abortion virus and two viral isolates designated as equine influenza virus suggested the desirability of studying possible relationships of the abortion virus to the influenza viruses of man and swine. The viruses of mumps and Newcastle disease were included because of their close biological relationship to the influenza viruses.[7, 8] A previous study by Jones and Maurer[23] demonstrated that serum of horses recovered from equine influenza did not neutralize the infectivity for mice of the human A or B viruses or swine influenza virus. The investigations presented here consist of neutralization tests in hamsters with hamster-adapted abortion virus, neutralization tests in embryonating chicken eggs with embryo-adapted mumps, Newcastle disease, and influenza (MNI) viruses, protection tests in hamsters immunized against the MNI viruses and challenged with the abortion virus, and hemagglutination (HA) and hemagglutination-inhibition (HI) tests employing MNI viruses, anti-abortion virus and anti-MNI virus serums.

MATERIALS AND METHODS

Viral Strains.—The viruses of the MNI group were the PR8 strain of influenza A virus, the FM1 strain of influenza A′ virus, the Lee strain of influenza B virus, the Shope-15 strain of swine influenza virus (SIV), the Henle-Enders strain of mumps virus and the Roakin strain of Newcastle disease virus (NDV). In addition, the JJ strain[15] of influenza C virus and the newborn pneumonitis virus (type Sendai),[17] which is tentatively considered as influenza D, were included. Except for the JJ strain which was propagated by amniotic inoculation of chicken embryos, each of the MNI viruses was adapted to growth in the allantoic sac of chicken embryos.

The Ky-D strain of hamster-adapted abortion virus was selected as representative of the agents presently known as equine abortion virus and described by Jones *et al.*[29] and Manninger[24] as equine influenza virus.

Preparation of Stock Virus.—For the PR8, FM1, Lee, SIV, and Sendai strains, 0.05 cc. of a 10^{-2} dilution of allantoic fluid virus was injected intra-allantoically into 10-day-old chicken embryos. After further incubation for 48 hours at 39 C., the living embryos were refrigerated overnight and the allantoic fluids were harvested. The Roakin strain was handled similarly, except that allantoic fluids were harvested from embryos that died 44 to 48 hours after inoculation. The JJ

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virus was similarly inoculated into the amniotic cavity of 10-day-old embryos. Postinoculation incubation was at 35 C. for 72 hours. The embryos were refrigerated overnight, and the amniotic fluid harvested. The mumps virus was injected intra-allantoically into 7-day-old embryos, which were then incubated for six days at 39 C., refrigerated overnight and the allantoic fluids harvested. The infective fluids were stored at —20 C. until used. The HA titers of the viruses used for the experiments described subsequently were 1:160 for PR8, Lee, and JJ, 1:320 for mumps, 1:640 for FM1, SIV, and Sendai, and 1:2560 for Roakin NDV.

Anti-Viral Serums.—For the MNI viruses, chickens 8- to 10-weeks-old that had no prior experience with NDV were given four intravenous injections of 1.0 cc. of allantoic or amniotic fluid virus at five-day intervals. The NDV was killed by addition of 0.02 per cent formalin. Five days after the last injection, the chickens were exsanguinated for serum collection. The serums were preserved with merthiolate, 1:5000, and stored at 4 C.

Anti-Ky-D strain serum was prepared from virus that had been maintained in horses by inoculation of equine fetuses. Lung tissue from an infected fetus was ground in a Waring blendor to make a 20 per cent suspension, and the supernatant fluid obtained after ten minutes of horizontal centrifugation at 2,000 relative centrifugal force (r.c.f.) was used for immunizing rabbits. The rabbits were given four intraperitoneal injections of 10 cc. of virus suspension at four-day intervals, and the serum was collected seven days after the last injection. The serum was filtered and stored at —20 C.

Normal serums were obtained from healthy chickens or rabbits and preserved and stored similarly.

In Vitro Neutralizations.—Anti-MNI virus serums were tested for ability to neutralize the abortion viruses by using hamsters as the indicator. The age of the hamsters varied from 5 to 6 weeks. The antiserums were inactivated at 62 C. for 30 minutes, and equal parts of undiluted serum and appropriate decimal dilutions of virus were mixed and incubated one hour at room temperature before intraperitoneal injection into 4 hamsters for each dilution. The dose for each hamster was 0.4 cc. for the serum-virus mixture and 0.2 cc. for the virus titrations. The hamsters were caged individually. Specific deaths occurring from the second through the seventh days were used for calculating 50 per cent lethal dose ($l.d._{50}$) titers according to the method of Reed and Muench.[20]

Neutralizations with the MNI viruses against their homologous anti-serums and anti-abortion virus serum employed chicken embryos as the indicator. The serums were inactivated and the serum-virus mixtures incubated as described above. Each of four embryos per dilution was inoculated intra-allantoically with 0.1 cc. of serum-virus mixture and 0.05 cc. for the virus titrations. End-points for titrations of NDV were based on embryo mortality from the second to the fifth days after inoculation. End points for other viruses of the MNI group were based on 50 per cent infectious doses ($i.d._{50}$) after the method of Hirst.[18] For the PR8, FM1, Lee, SIV, and Sendai viruses the embryos were incubated 48 to 60 hours after inoculation, refrigerated overnight, and the allantoic fluid was harvested from each embryo and tested for HA. A similar procedure was used for mumps virus, except that embryos were inoculated when 7 days old and were incubated six days before fluids were harvested. Fifty per cent end points were determined by the method of Reed and Muench.[20] Because titrations were not accurately reproducible, neutralizations in embryos were not made with the influenza C virus.

Hemagglutination Test.—Starting with a 1:5 dilution of amniotic or allantoic fluid virus, twofold dilutions were made in 0.25 cc. volumes of physiological saline contained in 13 x 100 mm. tubes. Chicken red blood cells (r.b.c.), 0.25 cc. of 1 per cent suspension, were added to each dilution. The agglutination patterns were ascertained after 40 minutes at room temperature for each virus, except JJ and Sendai for which the tests were conducted at 4 C. The highest dilution of virus causing complete agglutination was considered one hemagglutination (HA) unit.

Hemagglutination-Inhibition Tests.—Beginning with a 1:5 dilution of serum, serial twofold dilutions were made in 0.25 cc. volumes of physiological saline. Five HA units of virus contained in 0.25 cc. of physiological saline were added to each dilution. After ten minutes, 0.25 cc. of 2 per cent chicken r.b.c. was added to each dilution. The agglutination patterns were observed after 40 minutes at temperatures corresponding to those employed for the HA tests. The highest dilution of serum causing complete inhibition of agglutination was taken as the end point.

Immunization and Challenge of Hamsters.—For the MNI group, 0.5 cc. of allantoic or amniotic fluid virus was injected intraperitoneally at four-day intervals into hamsters 4 weeks old until four doses were given. Seven days after the last injection the hamsters were challenged by intraperitoneal injection of 0.2 cc. of decimal dilutions of abortion virus that were selected to encompass the $l.d._{50}$ titer of the virus. At the same time, serum from 5 immunized hamsters was collected and pooled for HI tests. Serum was obtained similarly from normal hamsters. Hamsters were immunized similarly with 0.5 cc. doses of heat-killed abortion virus, consisting of a 10 per cent suspension of infected hamster liver.

RESULTS

The *in vitro* neutralization and hemagglutination-inhibition tests are summarized (table 1). With the anti-abortion virus serum, there was no neutralization of infectivity for embryos of PR8, FM1, Sendai,

TABLE 1—In Vitro Neutralization and Hemagglutination-Inhibition (HI) Tests with Specific Antiserums and the Viruses of Mumps, Newcastle Disease, Influenza, and Equine Abortion

Virus	Chicken embryo $l.d._{50}$* of MNI** virus neutralized by anti-abortion virus serum	Hamster $l.d._{50}$* of abortion virus neutralized by MNI antiserums	HI activity of anti-abortion virus serum	Chicken embryo $l.d._{50}$* of MNI virus neutralized by homologous antiserums	HI titer† of homologous antiserums
Inf A, PR8	0.0	0.0	—	6.0	5120
Inf A', FM1	0.0	0.0	—	6.8	5120
Inf B, Lee	0.5	0.0	—	5.5	2560
Inf C, JJ	....	0.0	—	....	5120
Sendai‡	0.0	0.0	—	7.5	2560
Swine inf, S-15	0.3	0.0	—	7.3	1280
Mumps, Henle-Enders	0.0	0.0	—	5.5	1280
Newcastle, Roakin	0.0	0.0	—	6.3	2560

* Expressed as logarithm to base 10. Values for Newcastle disease virus are embryo $l.d._{50}$; ** mumps, Newcastle, influenza viruses; † highest serum dilution causing complete inhibition of agglutination by 5 hemagglutinating units of virus; ‡ tentatively considered to be influenza D virus.

mumps, or NDV. The results indicated neutralization of 0.5 log dilution of Lee and 0.3 log dilution of SIV.

The anti-MNI virus serums had no neutralizing activity against hamster-adapted equine abortion virus. When tested against the homologous virus, the anti-MNI serums had virus-neutralizing titers varying from $10^{5.5}$ to $10^{7.5}$ $i.d._{50}$ and HI titers varying from 1:1280 to 1:5120 (table 1).

The anti-abortion virus serum was devoid of HI activity against the MNI group of viruses.

Serum from hamsters immunized against PR8, FM1, and Lee had HI titers of 1:1280 against their homologous virus (table 2). Serum from hamsters immunized against JJ, SIV, mumps and NDV had HI titers of 1:640, and that from hamsters immunized against Sendai had a titer of 1:320. Serum from normal hamsters of the same age had no HI activity against the MNI viruses except inhibition at the 1:5 dilution for PR8 and at the 1:10 dilution for mumps virus.

The $l.d._{50}$ titers obtained in hamsters immunized against the MNI viruses and in comparable normal hamsters challenged with equine abortion virus are presented in table 2. Compared to the control titrations, the results indicated no protection against abortion virus by immunization against PR8, JJ, or Sendai. The differences of $l.d._{50}$ titers between the control hamsters and those immunized against FM1, Lee, SIV, mumps, and NDV varied from 0.3 to 0.7 log dilution.

DISCUSSION

Evidence of *in vitro* neutralization of viruses of the MNI group by anti-abortion virus serum was obtained only for Lee and SIV. The values of 0.3 and 0.5 log dilution for Lee and SIV are within the limits of variation for the procedure used in titrating the viruses of the MNI group, and therefore have no immunological significance. The homologous virus-neutralizing titer of the anti-abortion virus serum exceeded 10^6 hamster $l.d._{50}$ of the virus. Hemagglutination by viruses of the MNI

TABLE 2—Hemagglutination-Inhibition (HI) Responses of Hamsters Immunized Against Viruses of Mumps, Newcastle Disease, and Influenza, and Their Susceptibility to Equine Abortion Virus

Virus	Homologous HI titers of serum from immunized hamsters†	HI titer of serum from normal hamsters†	$l.d._{50}$ titers** of abortion virus	
			In hamsters immunized against MNI* viruses	In normal hamsters
Inf A, PR8	1280	5	7.5	7.3
Inf A', FM1	1280	—	7.0	7.3
Inf B, Lee	1280	—	6.8	7.3
Inf C, JJ	640	—	7.7	7.5
Sendai‡	320	—	7.0	6.5
Swine inf, S-15	640	—	6.6	7.3
Mumps, Henle-Enders	640	10	6.8	7.3
Newcastle, Roakin	640	—	6.7	7.3

* Mumps, Newcastle, influenza; ** expressed as logarithm to base 10; † highest serum dilution causing complete inhibition of agglutination by 5 hemagglutination units of virus; ‡ tentatively considered to be influenza D virus.

group was not inhibited by the same anti-abortion virus serum.

There was no neutralization of hamster-adapted abortion virus by antiserums for viruses of the MNI group. These antiserums had homologous virus-neutralizing titers varying from $10^{5.5}$ to $10^{7.5}$ $i.d._{50}$ and HI titers of 1:1280 to 1:2560.

Hamsters injected intraperitoneally with allantoic or amniotic fluid containing viruses of the MNI group were immunized, as indicated by the development of HI antibody to titers of 1:320 to 1:1280 against the homologous virus. However, such hamsters were not protected against challenge with the equine abortion virus. Titration of the abortion virus in hamsters immunized against MNI viruses and in comparable control hamsters indicated protection indexes of 0.3 to 0.7 log dilution, which are within limits of variation for the titration procedure.[13] Hamsters immunized similarly against heat-killed abortion virus were protected against 10^6 $l.d._{50}$ of abortion virus. Serum from normal hamsters had no significant HI activity against the MNI viruses. Since the equine influenza virus of Jones *et al.*[20] has been shown to be identical with equine abortion virus,[8] the experiments reported here confirm their report[21] that serums of horses recovered from equine influenza did not neutralize the A, B, or swine influenza viruses. The present study included viruses influenza A, influenza A′, influenza B, influenza C, Sendai which is tentatively designated as influenza D,[17] swine influenza, mumps, and Newcastle disease. There is no serological relationship of the equine abortion virus to the MNI group.

In addition to being serologically distinct, other differences exist between the abortion virus and the viruses of the MNI group. In the equine fetus, the abortion virus induces development of intranuclear inclusion bodies in bronchial epithelium, alveolar cells of the lungs, hepatic cells, endothelial cells, and others.[24] The lesions suggest pantropic properties, with the most pronounced lesions being present in the lungs and liver. Intranuclear inclusion bodies have not been described in tissues of young or adult horses infected with the abortion virus. However, in tissue culture preparations, many tissues from adult horses were infected by the virus and developed typical inclusion bodies.[28, 29] Intranuclear inclusions occur in the hepatic cells of infected hamsters and in the chorioallantoic membrane and liver of infected chicken embryos.[10-12] Intranuclear inclusion bodies are not characteristic lesions in infections caused by the influenza viruses and NDV. Johnson and Goodpasture[18] have reported cytoplasmic inclusions in acinar cells of the parotid gland in mumps infection of monkeys, but specificity of the lesion was not proved.

The known natural host range for the abortion virus is limited to the Equidae. Laboratory hosts in which infection has been confirmed are the hamster and the chicken embryo. Presently, the abortion virus has been propagated in chicken embryos only after adaptation to hamsters. The human and swine influenza viruses may be propagated in chicken embryos directly from the natural host, particularly with amniotic inoculation.[1, 27, 31, 33] The A, B, and swine influenza viruses are infective for ferrets, mice, and hamsters.[1, 32, 33] Influenza C virus is infective for ferrets and hamsters but not for mice.[1, 27] The equine abortion virus was not successfully passed in ferrets[20, 23] and is not infective for mice. Mumps virus is infective for suckling mice by intracerebral inoculation.[22] The Sendai virus is infective for mice, ferrets, and hamsters.[17]

SUMMARY

The possibility of serological relationship of the equine abortion virus to the influenza A, influenza B, influenza C, Sendai, swine influenza, mumps, and Newcastle disease viruses was studied by serum-virus neutralization, hemagglutination-inhibition, and protective immunization techniques.

Anti-abortion virus serum did not neutralize infectivity for chicken embryos or inhibit agglutination of chicken red blood cells by viruses of the mumps, Newcastle disease, and influenza group.

Anti-mumps, Newcastle disease, and influenza virus serums did not neutralize infectivity of hamster-adapted equine abortion virus.

Hamsters immunized against the mumps, Newcastle disease, and influenza viruses were not protected against the equine abortion virus.

The results show that equine abortion virus is serologically and immunologically

distinct from viruses of the mumps, Newcastle disease, and influenza group. Differences in the host range and specific lesions have been discussed.

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Agglutination of Horse Erythrocytes by Tissue Extracts from Hamsters Infected with Equine Abortion Virus

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EQUINE virus abortion was first described by Dimock and Edwards.[2]

Transmission experiments and clinical and pathological features of the disease were reported by Dimock *et al.*[3] and by Dimock *et al.*,[4] respectively. Doll *et al.*[7] adapted the virus to hamsters, and the hamster-adapted virus in turn was propagated in chicken embryos.[8] Other studies indicated that equine abortion virus and isolates described as equine influenza virus are closely related if not identical.[5]

Prior to the adaptation of the abortion virus to hamsters, extracts of equine fetal tissues that contained abundant intranuclear inclusions were employed in hemagglutination (HA) studies. Red blood cells (r.b.c) from horses, cattle, swine, sheep, mice, rabbits, chickens, guinea pigs, and man were tested. All trials gave negative results. The high content of virus in tissues of infected hamsters, often reaching 5×10^6 lethal dose $(l.d.)_{50}$ per gram, suggested the desirability of re-examining the hemagglutinative potentialities of the abortion virus.

This paper describes attempts to obtain agglutination of certain mammalian erythrocytes by hamster-adapted equine abortion virus and the resulting observation of an agglutinin, not neutralized by specific anti-equine abortion serum, common to certain tissue extracts from hamsters infected with abortion virus but not present in extracts prepared similarly from normal hamsters.

MATERIALS AND METHODS

Viral Strains.—Three strains of hamster-adapted equine abortion virus, Kentucky-A, Kentucky-B, and Kentucky-D, were used in these studies. The term "strain" means only that the culture is an isolate from a particular outbreak and not a defined entity within the species.

Kentucky-A was obtained from a naturally occurring outbreak of virus abortion in January, 1953. Virus from the lung of an aborted fetus was adapted to suckling hamsters and subsequently carried through numerous passages in hamsters 4 to 6 weeks old. The material used in these studies came from the 181st hamster passage.

Kentucky-B originated from an equine fetus that was aborted in February 1951. The virus was passaged by direct inoculation of an equine fetus *in utero* in March, 1952, and again in March, 1953.[5] Virus from the 1953 passage was adapted to hamsters, and the material used in the following studies was from the 115th hamster passage.

Kentucky-D came from an equine fetus that was aborted in February, 1952. It was passed twice by direct inoculation of equine fetuses *in utero* in February, 1953. Second-passage virus was adapted to suckling hamsters and subsequently carried through numerous passages in older hamsters. Material used in these experiments was derived from hamster passages 124 to 130.

Hamsters 4 to 6 weeks old which died after intraperitoneal inoculation with 1.0 ml. of an antibiotic-containing supernatant from a 10 per cent saline homogenate of infected liver served as the source of the virus. Death usually occurred between 18 and 36 hours after inoculation.

Erythrocytes.—Blood of various mammalian species was collected in saline containing 2.0 per cent sodium citrate. The red blood cells were washed three times in saline and made up by volume to a 1.0 per cent suspension in saline for HA tests.

Extraction of Tissues for Hemagglutinin.—Various tissues were collected from hamsters dying from injection of equine abortion virus and pools made of like tissues. A 10 per cent saline homogenate of each pool was made by grinding thoroughly in a TenBroeck grinder. The homogenates were centrifuged at 2,000 g in an International refrigerated centrifuge (model PR-1). Uninfected hamsters were killed and the tissues extracted in a like manner. The supernatant was used for HA studies and was considered a 1:10 dilution of the tissue.

Hemagglutination Test.—Starting with a 1:10 dilution of saline tissue extract supernatant, serial twofold dilutions were made in 13 x 100 mm. tubes containing 0.25 ml. physiological saline.

Red blood cells, 0.25 ml. of a 1.0 per cent saline suspension, were mixed with each dilution. The patterns were ascertained after standing for approximately one hour as indicated by the saline control tube. The end point was considered as the last tube to show complete agglutination (1 HA unit).

Hemagglutination-Inhibition Test.—Starting with a 1:5 dilution of serum, serial twofold dilu-

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tions were made in 0.25-ml. volumes of physiological saline contained in 13 x 100 mm. tubes. Ten HA units of tissue extract, contained in 0.25-ml. volume, were mixed with each dilution and after ten minutes 0.25 ml. of a 1.0 per cent suspension of horse r.b.c. was added to each tube. The patterns were ascertained after standing for 75 minutes at room temperature.

Complement-Fixation (c.f.) Test.—The technique employed has been described previously.[8]

Hamster Lethal Dose ($l.d._{50}$) Determinations.—Tenfold dilutions of tissue extracts were made in saline and 4 hamsters 4 to 6 weeks old were inoculated intraperitoneally with each appropriate dilution. The $l.d._{50}$ titer was calculated by the method of Reed and Muench.[10]

RESULTS

Testing Mammalian Erythrocytes for Susceptibility to Agglutination by Liver Extracts from Normal and Infected Hamsters.—Red blood cells from the chicken, hamster, guinea pig, mouse, sheep, hog, rabbit, cow, and horse were subjected to HA tests at 4, 27, and 37 C. Red blood cells from the chicken, guinea pig, sheep, hog, and cow were not agglutinated. Agglutination of mouse and rabbit erythrocytes was very erratic, but horse r.b.c. were agglutinated consistently to a high titer at each temperature. Extracts of livers from uninfected hamsters did not agglutinate horse r.b.c. Subsequently, a number of horses were bled for determining the effect of the individual on susceptibility of the equine r.b.c. to agglutination (table 1). Red blood cells of all horses appear equally susceptible to agglutination by liver extracts of hamsters infected by three viral strains. The agglutination patterns produced by these extracts were extremely stable as they remain unchanged on standing at 27 C. for at least 12 hours. Also, when the tubes were shaken and the new patterns observed, the titers approximated the original readings.

Effect of Immune Serum on Agglutination of Horse Erythrocytes.—Antiserums of rabbit origin were unsatisfactory since they, as well as normal rabbit serums, agglutinated horse r.b.c. to a high titer. Normal horse serum and serum from horses immunized by natural infection with equine abortion virus were also employed. Normal and immune serum behaved similarly in that no appreciable inhibition of agglutination occurred. Serums from horses that had received massive doses of a 30 per cent formolized liver homogenate prepared from infected hamsters caused considerable inhibition of agglutination. Serums from chickens immunized against Newcastle disease virus and the FM1, PR8, Lee, and Shope-15 strains of influenza virus did not inhibit agglutination.

TABLE 1—Agglutination Titers of Liver Extracts from Hamsters Infected with Equine Abortion Virus for Equine Red Blood Cells

Source of r.b.c.	Strain of Virus		
	Ky-A	Ky-B	Ky-D
Horse No.			
1	2560*	1280	2560
2	5120	1280	2560
3	2560	640	2560
4	2560	1280	2560
5	2560	1280	2560
6	2560	640	2560
7	2560	640	2560
8	2560	1280	2560
9	2560	640	2560
10	2560	320	2560

* Expressed as reciprocal of initial dilution of tissue.

Distribution of Hemagglutinin in Hamster Tissues.—Six hamsters, 4 to 6 weeks old, were inoculated intraperitoneally with the Ky-D strain of abortion virus. Certain tissues and urine were collected from dead hamsters and pools of like materials made. Blood, urine, and supernatants from the 10 per cent tissue homogenates were tested for their capacity to agglutinate horse r.b.c. (table 2). Urine and extracts of liver, lung, and spleen agglutinated r.b.c. to a rather high titer while blood and extracts of brain, kidney, heart, and skeletal muscle caused minor or no agglutination. Except for urine, which contained smaller amounts of virus, each preparation had a high hamster $l.d._{50}$ content.

Adsorption of Hemagglutinin by Horse Red Blood Cells.—Three trials were made. Absorption of 10 per cent liver extracts with equal volumes of 1.0 per cent horse r.b.c. for 30 minutes reduced the titer very

TABLE 2—Distribution of Hemagglutinin (HA) in Hamsters Infected with Equine Abortion Virus

Extract	HA titer	Hamster $l.d._{50}$ content/Gm.
Liver	1280*	5 x 9.5**
Lung	640	5 x 8.6
Spleen	640	5 x 9.5
Brain	0	5 x 7.5
Kidney	0	5 x 8.3
Heart	40	5 x 8.5
Blood	0	5 x 8.0
Urine	640	5 x 4.6
Muscle, skeletal	20	5 x 7.0

* Expressed as reciprocal of initial dilution of hamster material.

** Expressed as logarithm to base 10.

TABLE 3—Effect of Heating at 56 C. on the Hemagglutinating Capacity of Liver Extracts from Normal and Virus-Infected Hamsters

Liver extract	Time in minutes at 56 C.						
	0	5	10	15	30	60	120
Strain Ky-A	2560*	40	40	80	80	80	80
Strain Ky-B	320	40	40	80	80	80	80
Strain Ky-D	1280	40	20	80	80	80	80
Normal	0	80	160	160	160	160	160

* Expressed as reciprocal of initial dilution of tissue.

little. Considerable reduction of the HA content occurred when 1.0 ml. of packed cells was incubated similarly with 5.0 ml. of extract. The agglutinin was absorbed completely in equal volumes of tissue extract and packed cells.

Effect of pH and Heating on HA Activity.—For the pH study, hamster livers were extracted in saline buffers at pH 6, 7, 8, and 9 and each supernatant was subjected to HA tests at the corresponding pH. Agglutination to a high titer occurred with each extract.

Liver extracts prepared from normal hamsters and from hamsters infected with viral strains Ky-A, Ky-B, and Ky-D were heated in a 56 C. water bath. After 5, 10, 15, 30, 60, and 120 minutes, appropriate samples were collected and tested for HA activity (table 3). Unheated extracts of livers of infected hamsters agglutinated equine r.b.c. to a high titer while unheated extracts from uninfected hamsters were without this capacity. After heating for five minutes, the HA titers of viral supernatants were greatly reduced and remained approximately at the same level throughout the test period. Normal liver extracts which had been heated produced similar agglutination-like patterns of equal titer. The agglutinin produced as a result of viral infection apparently is destroyed after five minutes at 56 C. Agglutination-like patterns, however, were observed in the first three or four tubes with heated liver tissue extracts from either normal or infected hamsters. This reaction appears to be caused by partial coagulation of the extract by heat.

TABLE 4—Hemagglutinative (HA) and Complement-Fixing (CF) Activity of Filtrates from Seitz EK, Selas 02, and Ultrafine Filters *

Filtrate	HA	CF	Infectivity for hamsters
Seitz EK	0	+	+
Selas 02	+	+	+
Ultrafine filters			
Coarse	—	+	+
Medium	—	Incomplete	+
Dense	—	Incomplete	+
Very dense	—	—	—

* Schleicher and Schuell filters having porosities of 0.2 to 0.08, 0.08 to 0.05, 0.05 to 0.01, and 0.01 to 0.005 μ for coarse, medium, dense, and very dense filters, respectively.

Filtration and Centrifugation Studies.—Hamster liver extracts were passed through Seitz EK; Selas 02; and ultrafine filters (Schleicher and Schuell) coarse, medium, dense, and very dense grades having porosities 0.2 to 0.08, 0.08 to 0.05, 0.05 to 0.01, and 0.01 to 0.005 μ, respectively. These filtrates were tested for c.f. and HA activity (table 4). The ultrafine filtrates were tested for infectivity, but the Seitz and Selas filtrates were not, because on previous occasions such filtrates had hamster l.d.$_{50}$ titers approximately equal that of the unfiltered material. The Seitz and ultrafine filtrates had no HA activity. The Selas filtrate caused HA that was but slightly diminished from unfiltered material. The Selas, Seitz, and coarse ultrafine filtrates fixed complement; the medium and dense ultrafine filtrates produced incomplete fixation; and the ultrafine very dense filtrate was inactive. All filtrates were infective except that from the very dense filter.

Ten per cent hamster liver extract was centrifuged in the Spinco ultracentrifuge (model L, No. 40 rotor) at 105,400 g for 90 minutes. The supernatant had no HA activity; however, when the sediment was resuspended full HA activity was observed. The supernatant fixed complement and was infective for hamsters.

Time Development of Hemagglutinin.—Hamsters were inoculated intraperitoneally with $10^{7.5}$ l.d.$_{50}$ of Ky-D strain of abortion virus. At 3, 6, 9, 12, 15, 18, and 21 hours after inoculation 5 hamsters were killed and their livers pooled and extracted. The c.f. capacity, the hamster l.d.$_{50}$ and the HA activity were determined. Complement fixation and HA activity first appeared at nine hours and each remained almost constant throughout the test period. These results will be more fully described in another paper.[1]

DISCUSSION

The hemagglutinin present in certain tissue extracts of hamsters infected with equine abortion virus appears at first somewhat unique in that specific antiserum

with high neutralizing capacity does not inhibit agglutination. Even extracts whose infectivity is completely neutralized by such serums still agglutinate horse r.b.c. Serum of horses immunized with formolized liver tissue of infected hamsters, however, caused inhibition of agglutination. Unfortunately, serums from horses immunized with similar preparations of uninfected hamsters were not available for comparative study. These studies suggest that agglutination of horse r.b.c. is caused by some factor produced as a result of pathological alteration of the tissues and that this factor is distinct from the virus particle.

Evidence that the infection is responsible for the production of the hemagglutinin is demonstrated by the complete lack of HA activity in normal tissue extracts. Further evidence that the hemagglutinin is not intimately associatel with the viral particle is that the hemagglutinin does not pass through the Seitz EK filter while most of the infectivity does. The Selas 02 filtrate, however, contains both the infectivity and hemagglutinin. Centrifugation removes the hemagglutinin without removing infectivity. These studies indicate that the hemagglutinin is a rather large particle and that it is adsorbed to the Seitz filter which accounts for its inability to appear in the filtrate. The high hamster $l.d._{50}$ content of blood, brain, kidney, and heart and the almost total absence of HA activity is another indication that the hemagglutinin is distinct from the infective particle.

Adequate studies have not been done on hamsters infected with equine abortion virus to assist an explanation of why some tissues have agglutinin and others do not. It is conceivable that the high viral content of the kidney, muscle, brain, and heart is a result of viremia and not due entirely to multiplication. It is possible that the hemagglutinin is present, for the most part, in those tissues where most of the multiplication occurs.

Summary

A hemagglutinin for horse erythrocytes is described. It is common to certain tissue extracts from hamsters infected with hamster-adapted equine abortion virus but is not present in extracts prepared similarly from uninfected hamsters.

Red blood cells from all horses appear equally susceptible to agglutination.

The hemagglutinin is present to a high titer in liver, lung, spleen, and urine. Little, if any, is present in brain, kidney, skeletal muscle, and heart. All tissues possessed a high hamster $l.d._{50}$ content.

Specific anti-equine abortion serum did not inhibit agglutination; however, serum from horses that were injected subcutaneously with a formolized liver homogenate prepared from infected hamsters inhibits agglutination. This suggests that the hemagglutinin and virus particle are not intimately associated.

The hemagglutinin can be adsorbed by horse red blood cells; its activity is destroyed after five minutes at 56 C.; it is completely removed by centrifuging at 105,400 g for 90 minutes; it does not pass through the Seitz EK filter but does pass through the Selas 02 filter, whereas the virus passes through each without appreciable reduction of titer.

The hemagglutinin is detectable in hamster tissues approximately nine hours after intraperitoneal inoculation with $10^{7.5}$ $l.d._{50}$ of virus.

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Studies on the Depression of Radioiodine Uptake by the Thyroid After Phenothiazine Administration
II. Effect of Phenothiazine on the Horse Thyroid

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PHENOTHIAZINE N. F. (green) was found by Talmage *et al.*[1,2] to suppress the uptake of radioiodine by the thyroid gland of a number of species. Two possible mechanisms were presented to explain these observations: (1) iodine, an impurity in commercial phenothiazine preparations, saturated the thyroid gland and diluted the radioactive iodine with the stable isotope or (2) some agent other than iodine in commercial phenothiazine truly inhibited thyroid function which would result in hypothyroidism. The first suggested mechanism may not be necessarily detrimental to hormone synthesis or iodine accumulation by the thyroid if the levels of iodine are sufficiently low.[3] At higher levels, iodine would not only depress or suppress thyroidal uptake of I-131, but also would inhibit thyroid hormone production.[4] The evidence presented in the previous paper of this series[5] indicated that the iodide ion in phenothiazine N. F. (green)* was the primary agent responsible for the observed thyroid effects.

It was deemed necessary to determine whether the levels of iodine encountered in therapeutic doses of phenothiazine were sufficient to depress thyroid function. Whereas an induced hypothyroidism may be a distinct advantage in some phases of animal husbandry, it would be contraindicated in raising Thoroughbred horses. The purpose of the present study was to evaluate the effects of phenothiazine in the therapeutic dose range on the thyroid physiology of the horse.

METHODS AND EXPERIMENTAL DATA

Effects of Single High Level or Continuous Low Level Therapeutic Doses of Phenothiazine on Thyroid Uptake of Radioiodine in Stabled Horses.—Experiment 1.—Seven stabled horses were maintained on a hay ration having less than 22 μg. of iodine per 100 Gm. for a month prior to treatment. The horses were allotted to groups so that 3 horses served as controls, 2 were given an oral dose of 25 Gm. of phenothiazine N. F. (green) and 2 were given 0.103 Gm. of potassium iodide. The total iodine content of the KI and the green phenothiazine was equivalent. Twenty-four hours after treatment each horse was intravenously injected with 30 μc. of carrier-free I-131. The I-131 radioactivity accumulated by the thyroid was periodically measured externally with a sensitive scintillation probe attached to a count rate meter. A distance of 20 cm. between the probe and the thyroid area was chosen because small movements of the probe or animal at this distance did not appreciably change the readings.

As in other species, it was found that phenothiazine N. F. (green) depressed I-131 uptake by the horse thyroid gland (fig. 1). Under these conditions, potassium iodide appeared more effective than phenothiazine in decreasing the accumulation of radioiodine. In contrast, the chick[5] and the rat[6] responded about the same to both KI and phenothiazine N. F. (green). These differences tended to (1) give support to the suggestion by Talmage *et al.*[2] that various species respond differently to phenothiazine N. F. (green) or (2) indicate variations in experimental conditions other than species differences. Subsequent experiments bear out the latter postulate.

Experiment 2.—The effect of continuous daily low level dosage of phenothiazine on thyroid accumulation of radioiodine was also examined. After receiving 2 Gm. of phenothiazine N. F. (green) per day orally for 18 days, 2 horses were intravenously injected with 40 μc. of carrier-free I-131. Two nontreated control horses were simultaneously injected with I-131. The thyroid uptake was measured externally, as previously described, at various time intervals for 12 days after I-131 administration. The thyroid uptakes were calculated as percentage of a standard I-131 dose measured similarly as the thyroid. No depression in thyroid uptake was observed under these conditions (table 1).

This preliminary experiment indicated

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* Impure commercial phenothiazine (N.F.) with greenish color as distinguished from a purified yellow phenothiazine not usually offered commercially.

that a continuous therapeutic dose of phenothiazine was unable to depress the accumulation of radioiodine by the horse thyroid.

TABLE 1—Effect of Continuous Daily Low Level Phenothiazine Treatment on Uptake of Radioiodine by the Horse Thyroid

		Uptake of Radioiodine (% of dose)			
		Hours after I-131 administration			
Horse	Treatment	6.5	23	120	288
1	Control	5.5	7.4	7.6	6.9
2	Control	5.8	7.2	6.5	6.5
3	Phenothiazine* N.F. (green)	7.3	8.3	8.5	6.8
4	Phenothiazine N.F. (green)	5.2	7.9	7.1	6.5

* Dose level: 2 Gm. per day 18 days before thyroid measurement and during course of thyroid measurement.

Effects of Phenothiazine on Blood Disappearance and Tissue Distribution of Radioiodine, and on Thyroid Histology in Stabled Horses.—Experiment 3.—Further comparison of the effects of phenothiazine and KI on the thyroid physiology of the horse included the study of the plasma disappearance and tissue distribution of intravenously injected I-131. Histological and autoradiographical observations of the thyroid glands from phenothiazine-treated animals were also made. Two horses, maintained on a low iodine hay ration, were given a single oral dose of either 25 Gm. of phenothiazine N. F. (green) or the equivalent iodine level of KI. Another horse served as control. The day following treatment, each animal was intravenously injected with 25 mc. of carrier-free I-131. Daily blood samples were taken until the horses were destroyed on the sixth day. Tissues were obtained for radioactive assay and the thyroids placed in formalin for future histological and autoradiographical preparations.

Close examination of the plasma disappearance curves (fig. 2) indicated that I-131 disappeared at a similar rate from the blood of the horses treated with either KI or green phenothiazine N. F. The control horse values fell parallel with the experimental levels for approximately 16 hours but declined much less rapidly thereafter. The disappearance of I-131 from plasma are dependent mainly on four metabolic processes, namely: (1) the uptake of inorganic I-131 by the thyroid, (2) movement of I-131 into tissues, (3) excretion of I-131 via the urine and feces, and (4) the release of I-131 labelled thyroid hormone from the thyroid back into the circulatory system. Processes (1), (2), and (3) are responsible for the rapid disappearance of I-131 immediately after dosage. The I-131 accumulated by the thyroid is incorporated into newly synthesized thyroid hormone and subsequently released from the thyroid in this bound form, *i.e.*, process (4). As indicated by the slope-changes in the

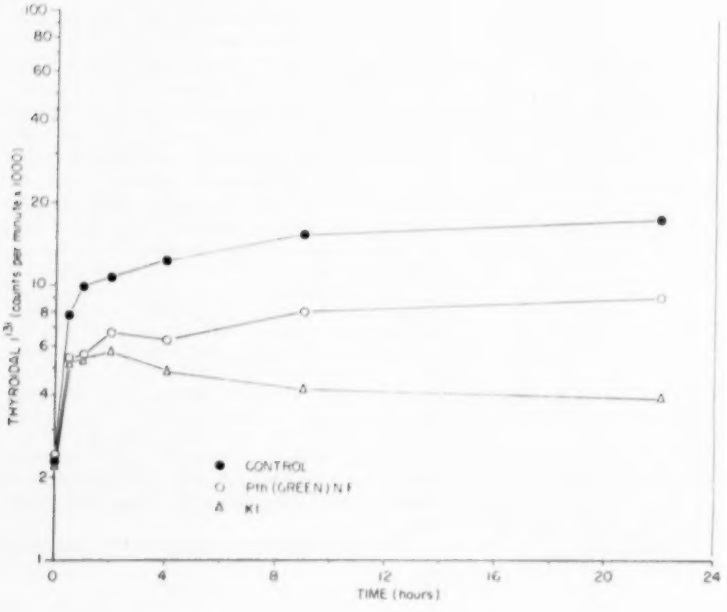

Fig. 1—The effect of phenothiazine and potassium iodide on the uptake of I-131 by the horse thyroid.

disappearance curves (fig. 1), bound radioiodine became a detectable part of total plasma I-131 approximately 22 hours following I-131 administration. The production of protein-bound I-131 was not inhibited in the dosed animals since the determined values for percentage of protein-bound (PB) I-131 were about 55 per cent in all animals four days after treatment. Because the thyroids of the treated horses accumulated much less I-131 than the control, the excess plasma inorganic I-131 rapidly disappeared via the excretory pathways. This would account for the quantitative differences in total I-131.

The distribution of I-131 in the thyroid gland, liver, spleen, kidney, brain, and adrenal glands is shown, as percentage of the injected dose (table 2). Aliquots of the tissues were counted in a deep-well scintillation counter. As was the case with the external thyroid measurements, potassium iodide and phenothiazine similarly depressed thyroidal uptake of I-131. The amount of I-131 in the examined tissues was also depressed to about the same degree by the administration of KI or phenothiazine N. F. (green). LeLoup [7] reported that thiouracil depressed the accumulation of I-131 in the thyroid of the dogfish, accompanied by the expected increase in urinary excretion of I-131 and an increase of I-131 content in other tissues. Any agent which specifically inhibited thyroidal I-131 uptake would not necessarily affect the I-131 taken up by other tissues. The quantitative similarity of the tissue distribution in the phenothiazine and KI horses suggested that the agent in the phenothiazine diluted the trace dose of I-131 as did KI, or adversely affected the iodine uptake by all tissues of the body. The former possibility is the more plausible.

TABLE 2—Effect of Potassium Iodide (KI) and Phenothiazine (PTH) N. F. (Green) on Tissue Distribution of I-131 in the Horse

Tissue sample	Percentage of I-131 dose in total organ		
	Control	KI	PTH
Thyroid	18.2	4.4	5.1
Liver	0.34	0.06	0.06
Spleen	0.02	0.008	0.006
Kidney	0.05	0.008	0.008
Brain	0.002	0.0006	0.0005
Adrenal	0.0008	0.0002	0.0002

Examination of the autoradiographs and histological preparations of the thyroids of the 3 horses revealed no changes attributable to treatment. All appeared normal, and thyroid hypertrophy of the thiouracil-type was not in evidence. The autoradiograms showed that, qualitatively, the distribution of I-131 in various compartments of the thyroid (*i.e.* colloid, acinar cells, etc.) was the same for the control and the experimental horses. These observations are in contrast to the effects of propylthiouracil which prevent the appearance of iodine in the colloid of the thyroid.[8]

Thyroid Uptake and Protein-Bound Iodine Lev-

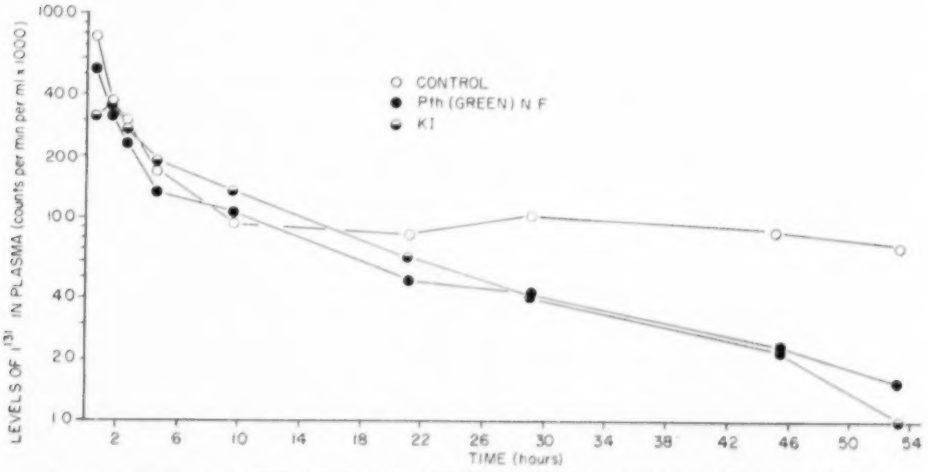

Fig. 2—The effect of phenothiazine (green) N. F. and potassium iodide on plasma I-131 levels in the horse.

els in Pastured Thoroughbreds.—True antithyroid drugs will eventually depress the systemic thyroid hormone level. Symptoms of hypothyroidism, such as decreased tissue respiration, obesity, edema, and lower metabolic rate, would then occur. The estimation of serum thyroid hormone levels was undertaken in order to ascertain whether phenothiazine N. F. (green) or purified phenothiazine N. F. treatment, in actuality, inhibited thyroid hormone synthesis in the horse. At the same time, the effect of these agents and KI on thyroidal I-131 uptake was determined in pastured horses (as compared to stabled horses in the previous section).

TABLE 3—Effect of Potassium Iodide and Phenothiazine on I-131 Thyroid Uptake and Serum Protein-Bound Iodine (PBI) Level in the Pastured Horse

Treatment	No. animals	Thyroid uptake of I-131 (% of dose)	Serum PBI (μg./100 ml.)
Control	8[1]	14.2 ± 3.1[2]	2.0 (1.6-2.7)[3]
KI	6	6.6 ± 0.4	.....
PTH (green)[4]	13	8.4 ± 0.6	2.5 (2.0-4.0)
PTH (purified)[4]	6	12.9 ± 1.3	2.1 (1.9-2.8)

[1] Average control values are from 2 pastured and 8 stabled horses; [2] mean ± standard error of the mean; [3] range of values in parenthesis; [4] PTH (green) = phenothiazine N.F. (green); PTH (purified) = phenothiazine N.F. (purified).

Experiment 4.—Twenty-seven pastured Thoroughbreds were placed in four groups (table 3) and for 30 days prior to test no iodized salt was accessible to these animals. Group 1 served as controls. Groups 2, 3, and 4 were given, orally, 0.103 Gm. of KI, or 25 Gm. of phenothiazine N. F. (green), or 25 Gm. of purified phenothiazine N. F., respectively, in a single capsular dose. Twenty hours after treatment, all horses were intravenously injected with 1 mc. of carrier-free I-131. The accumulation of radioiodine by the thyroid was measured externally with a portable scintillation dose rate meter for five days. Daily blood samples were obtained for serum protein-bound iodine (PBI) determinations. Serum PBI was determined by precipitating serum proteins with cold 10 per cent trichloracetic acid (TCA), washing the precipitate in TCA, and analyzing the precipitate for total iodine.

The thyroids of the control horses and horses receiving purified phenothiazine accumulated 14.2 per cent and 12.9 per cent of the injected I-131 dose, respectively, while the animals getting KI and green phenothiazine accumulated 6.6 per cent and 8.4 per cent of the dose, respectively (table 3). Both KI and phenothiazine N. F. (green) depressed thyroidal I-131 uptake by approximately 50 per cent. In this experiment, KI was no more effective than phenothiazine (green) in depressing thyroidal I-131 uptake. This suggested that the different response of the thyroid to KI vs. phenothiazine in stabled animals was due to different experimental conditions not discernible at this time.

The estimation of circulating thyroid hormone, as serum PBI, revealed no significant difference between those horses that received either of the phenothiazines and the controls (table 3). Although PBI is not quantitatively equivalent to serum thyroxine iodine (or tri-iodothyronine iodine), it has been used extensively as an estimation of thyroid hormone. These results indicate that the phenothiazines did not alter the synthesis of thyroid hormone in the horse.

DISCUSSION

Examination of data indicated that phenothiazine N. F. (green) and KI similarly affected the metabolic pattern of I-131 in the horse. Each reduced thyroidal I-131 accumulation to approximately the same extent and resulted in a similar tissue distribution and plasma disappearance of I-131. No evidence of an abnormal thyroid gland was apparent in the phenothiazine-treated horses as indicated by histological and autoradiographical examination. In addition, the horse serum PBI values were not depressed by phenothiazine administration.

Purified phenothiazine N. F., on the other hand, did not suppress the accumulation of I-131 by the thyroid. This is in agreement with the large animal studies of Talmage *et al.*[2] where external counting was employed. Later experiments by Nachimson *et al.*[6] and Wasserman *et al.*[5] indicated that purified phenothiazine N. F. caused a small but significant depression of thyroidal I-131 uptake in rats and chicks, respectively. In these latter studies, the thyroids were excised and the activity measured more exactly in a deep-well scintillation counter.

The present studies confirm the previous findings that the action of phenothiazine N. F. (green) is explainable on the basis of the available iodine present therein. Since the level of serum thyroid hormone (as PBI) was not depressed by either KI or the phenothiazines, the amount of iodine in these preparations was not sufficient to result in abnormal thyroid function. This is borne out by the early toxicity studies with phenothiazine where Eddy *et al.*[9] ob-

served no pathological changes in the rat thyroid due to the continuous ingestion of phenothiazine for 300 days. Recently Nachimson *et al.*[6] found no gross thyroid changes in the rat at dietary levels of 0.075 per cent phenothiazine although thyroidal I-131 uptake was reduced by one-half. Based on the data presented in this paper and the information cited from the literature, the conclusion is drawn that the metabolism of the horse thyroid is not significantly affected by a single therapeutic dose of phenothiazine N. F. (green) although a depression of I-131 uptake was demonstrated.

SUMMARY

1) The effect of phenothiazine on the thyroid gland of the horse was evaluated.

2) Single therapeutic doses of phenothiazine N. F. (green) were found to depress thyroidal I-131 uptake in the horse. Purified phenothiazine had no effect in this regard.

3) Low level doses, 2 Gm. daily, of phenothiazine N. F. (green) did not depress thyroid uptake of I-131 either on the eighteenth or thirtieth day of continuous administration.

4) Phenothiazine N. F. (green) and KI similarly affected the plasma disappearance curve and tissue distribution of I-131 in the horse.

5) Determination of plasma protein-bound iodine in the horse revealed no subnormal values due to phenothiazine treatment.

6) This study indicates that phenothiazine has little, if any, effect on thyroid function in the horse.

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The Static Intravascular Extracapillary Blood Volume of the Kidney of the Dog as Estimated by the Vinylite Corrosion Technique

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METHODS for the estimation of the blood volume of organs are essentially those used for the detection of blood volume of the total body. They involve the injection of a substance which will remain in the blood stream for a satisfactory period with subsequent determination of the dilution of that substance in the circulating blood. Blood volume determinations for organs have involved removal of the organ and analysis for content of the circulating blood. Such determinations result in an evaluation of the total volume of blood in the organ. There are no available techniques for evaluation of the blood volume of any particular portion of the vascular system of an organ (such as arterial system as compared to venous system).

The characteristics of the vinylite* corrosion technique are such that, upon careful standardization, the technique can afford a reasonably accurate direct means of determining intravascular extracapillary blood volumes for certain organs and the relative volumes of vascular or tubular systems in such organs. Vinylite injections do not enter capillary beds; thus, each vascular system can be evaluated independently. This paper reports the application of this technique to the estimation of the extracapillary blood volume of the kidney of the dog and the relative volume of the arterial and venous systems in the kidney.

METHODS

Twenty-six dogs were used in this study. Estimation of the total extracapillary blood volume of the kidney was accomplished on 23 dogs. Relative volumes of arterial as compared to venous circulations were estimated in 5 dogs. The dogs were obtained from a variety of sources and ranged in age from 6 months to 8 years. The procedure involved the preparation of vinylite corrosion casts of the arterial or the venous systems or both. This cast was weighed and the weight compared to the weight of a standard volume of the injection mass. This procedure gave an expression of volume of the vascular system in cubic centimeters. This value was converted to a value for the weight of a corresponding volume of whole blood and the latter was expressed as percentage of the total weight of the kidney.

Procedure.—The technique consisted of the following steps.

1) Both kidneys were removed. One was used for the preparation of microscopic sections for the evaluation of the normalcy of the kidneys. The other was cannulated.

2) The blood was removed by injection of physiological saline solution.

3) The saline was removed by the injection of air.

4) The kidney was dried by the use of paper towels. The cannulas were removed. The vessels were trimmed to standard lengths and all accessible peritoneum and perirenal fat was removed. The kidney was weighed.

5) The cannulas were replaced.

6) Acetone was injected to prepare the organ for the vinylite solution.

7) Air was used to force most of the acetone from the organ.

8) A standard solution of 12 per cent vinylite in acetone was used for the injection, the injection being accomplished by use of a glass syringe. A sample of vinylite was injected into a volume of water producing a mass of vinylite representing a known volume of the injection mass. This mass of vinylite accompanied the kidney preparation through all succeeding steps thus insuring uniform handling of the cast and the standard.

9) The kidney was corroded in crude concentrated hydrochloric acid. The cast was washed, adherent fat was removed by hot water or alkali or both and the cast was permitted to dry.

10) The cast was trimmed and weighed. The standard mass of vinylite was weighed. Before the casts were weighed they were examined by means of a dissecting microscope to determine the degree of completeness of injection. Those judged incomplete were discarded. In the majority of the cases, the casts used in this study were complete to the interlobular vessels and their primary branches. In a few of the arterial casts, glomeruli were revealed.

Calculations.—(1) The weight of the standard of vinylite, which represented 10 cc. of injection

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* Vinylite was obtained from the Bakelite Corporation, 30 E. 42nd St., New York 17, N. Y.

mass, divided by 10 gave an expression of the weight of 1 cc. of vinylite solution.

2) The weight of the cast divided by the weight of 1 cc. of vinylite solution represented the volume of the vascular system of the kidney.

3) The volume of the cast times 1.059 (specific gravity of dog's blood) equaled the weight of blood represented by the cast.[5]

4) The weight of the blood was added to the weight of the washed kidney (step 4 above). This weight was divided into the weight of the blood represented by the cast. The resultant figure (multiplied by 100) represented the static extracapillary blood volume of the kidney expressed as percentage of the total weight of the kidney.

Histological examination of the kidneys of the dogs in this series revealed minor degrees of fibrosis. There was no correlation between the degree of fibrosis and the volume of blood contained by particular kidneys; therefore, it was assumed that, within the limits of accuracy of the technique in question, minor renal changes encountered were of no significance to the determinations in this series of animals. No histological examination was made of kidneys of dogs used for determinations of relative volumes of arterial as compared to venous systems as both kidneys were required for the volume determinations.

RESULTS

Static Intravascular Extracapillary Blood Volume of the Kidney of the Dog.—The mean value for extracapillary blood volume for the 23 samples of this report was 20.4 per cent of the weight of the intact kidney. The range of values was 16.0 to 26.2 per cent with a standard deviation of 3.3 per cent of the total weight of the kidney.

Proportion of Blood on the Venous and Arterial Sides of the Renal Vascular System.—Estimation of volumes of venous as compared to arterial systems in 5 dogs resulted in average values of 35 per cent of the blood on the arterial side and 65 per cent on the venous side. The range of values was 27 to 43 per cent for the arterial side and 57 to 73 per cent for the venous side. Although these ranges are broad, it is to be noted that there is no overlap between groups.

DISCUSSION

For a more complete understanding of an organ, the anatomist must go beyond such considerations as form, position, relations, and microscopic structure. The volumes of tissue compartments; stroma vs. parenchyma, capillaries vs. larger vessels, arteries vs. veins, and the volume of duct systems are of significance in respect to this goal.

The observation that the volumes of the venous and arterial systems of the kidney of the dog exist in a 65 to 35 ratio illustrates the well known fact that there is more blood on the venous side of the circulation than the arterial side but extends this fact to the organ level.

Evaluation of Technique.—Since no values for the extracapillary blood volume of the dog's kidney exist, the above results can not be compared with published values. An evaluation of results must be made on the basis of consideration of the potential errors of each step in the procedure and their possible influence on the resultant values.

It would appear that few of the steps in the procedure could lead to an excessive value. The fact that the casts were prepared with the injection medium under greater than physiological pressures might be the most obvious source of potential error. However, the fact that the injection mass could pass no further than the finer precapillary vessels would constitute a barrier to excessive injection within the anatomical distribution of the vessels. Overextension of the vessels was limited by the general nondistensible nature of the kidney, distention being limited by the strong capsule. The fact that the kidney is normally subjected to reasonably high vascular pressures and is appreciably distended in the intact animal further decreases the likelihood of an excessively artificial situation being created in the injected kidney. Another factor which might contribute to a higher value would be the adherence of fat of the renal sinus of the cast and its subsequent inclusion in the weight of the cast. After removal from acid, the casts were treated with heated sodium hydroxide solution to remove the fat. The use of acetone might increase the resultant values since it denatures the tissue and frees small fragments. All the other steps in the process, if incompletely performed, would detract from the weights of the casts. Incomplete removal of the blood or any subsequent fluid would encroach upon the space available to the plastic and thus would serve to lessen the final results.

Possible Applications of Technique.—The vinylite corrosion technique presents the unique opportunity to study the vol-

umes of particular vascular or tubular systems. It might best be applied to organs which have the physical characteristics similar to kidneys since possible overextension of organs without a firm, unyielding capsule would produce a serious error. The technique might be applied to anatomical studies in search of differences or uniformity of volumes of vascular or tubular systems between species. Its application to more flexible organs (such as the liver) might be accomplished upon standardization of the pressures used in the injection technique.

Intriguing possibilities for the application of this technique exist in the field of pathology. Admittedly the process is rather crude and in its present form could not be expected to detect subtle changes in volumes brought about by some pathological processes; however, the influence of extensive changes (as those brought about by fibrotic processes) upon the static volume of an organ might be detected and subjected to description. This technique in combination with a method for evaluating total blood volume of an organ and a method (such as that used by Warren [2]) for estimation of the connective tissue of an organ would afford the anatomist or pathologist with data by which he could gain a more complete appreciation of the volume relationships of capillaries, other vessels, stroma, parenchyma, and duct systems of an organ. How much more meaningful the illustration of the effect of cirrhosis upon the blood volume of the liver as presented by McIndoe [3] would be if the observation could also be expressed in a semiquantitative fashion.

SUMMARY

A method for the estimation of extracapillary blood volume of a vascular system or the volume of a tubular system of an organ is described.

The average total static extracapillary blood volume of the kidney of the dog was found to be 20.4 ± 3.3 per cent of the weight of the kidney. Twenty-three animals were studied.

Of the extracapillary blood volume of the canine kidney, 35 per cent was found to be on the arterial side, 65 per cent on the venous side.

An evaluation of the technique and suggestions for its application are presented.

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Continuous Phenothiazine Therapy of Dogs Artificially Infected with *Ancylostoma Caninum*

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Ancylostoma caninum, the common hookworm of the dog, is cosmopolitan in distribution although the incidence varies in different parts of the world and in different localities of the United States.[1,2]

Cooperrider[1] reported that 85.7 per cent of the dogs in Georgia were infected. Whitney[2] reported that approximately 30 per cent of the dogs in Connecticut were infected. Rubin,[3] in a limited survey, found 72 per cent of the dogs in Oklahoma infected. The parasites are blood-sucking nematodes and one adult will ingest approximately 0.84 cc. of blood each 24 hours.[4]

Many methods of control of *A. caninum* have been advocated. Hoerlein[5] reported tests for efficiency of chemicals for sterilizing both artificially and naturally infected soils. Various chemicals were found to have some degree of efficiency in destroying ova on the ground but had disadvantages that limited their practical use. Todd[6] found that phenothiazine in small daily doses reduced the number of strongyle parasites. Mayhew[7] reported that phenothiazine in small continuous doses had an inhibitory effect on ova of certain parasites.

It has been shown that phenothiazine has an inhibitory effect upon the ova of *A. caninum* in fecal cultures when fed to dogs. Folse and Bailey[8] found that phenothiazine fed in small repeated doses caused a marked reduction of *A. caninum* eggs per gram of feces of dogs. Collier and Mack[9] and McNally[10] found that phenothiazine in relatively large doses produced anemia in dogs.

As a result of the preceding information an investigation was undertaken to determine the following.

1) The toxicity of phenothiazine to dogs when fed in small daily doses.

2) The effect of phenothiazine on the development of *A. caninum* in the host animal.

3) The effect of daily doses of phenothiazine on ova and larvae.

Materials and Methods

In each individual trial, a litter of dogs of mixed breed were used. The *A. caninum* used to artificially infect the dogs were cultured from feces of naturally infected dogs. Dogs infected with *A. caninum* only were used to furnish ova. The dogs used in the experiments were treated twice with normal butyl chloride, and held for at least three weeks after ova could no longer be demonstrated in the feces by a flotation technique. The normal blood values were determined by taking an arithmetic average of three consecutive daily examinations. After administration of larvae, flotation method fecal examinations were made on alternate days until ova appeared, then egg-per-gram counts were made weekly on feces. The ova from all dogs were cultured weekly. If larvae showed motility after escape from the egg shell, they were considered viable. Weekly erythrocyte counts, hemoglobin content, and hematocrit determinations were made throughout the experiment. At the end of each experiment, the dogs were destroyed and the intestinal content screened for *A. caninum*.

Experimental Data

Five dogs from a litter were used to determine the toxicity of phenothiazine with 1 dog, as a control, receiving none of the drug. The dogs fed 132 mg. and 198 mg. of phenothiazine per kilogram of body weight per day sometimes refused to eat the medicated feed; at these times, the drug was administered in gelatin capsules. The

TABLE 1—The Effect of Phenothiazine on Normal Dogs

Dog No.	Phenothiazine mg./kg./day	Normal values				Values at end of 60-day period			
		r.b.c.[1]	Hb.[2]	Hemat.[3]	Weight (lb.)	r.b.c.	Hb.	Hemat.	Weight (lb.)
1	17	5.13	12.5	48.0	19.0	5.2	13.5	55.0	20.0
2	66	6.86	14.0	55.0	15.0	6.8	14.25	53.0	18.0
3	132	6.44	13.5	53.0	17.5	5.1	12.5	39.0	19.5
4	198	6.56	14.5	58.0	13.0	4.95	10.25	31.0	11.0
5	0	5.5	12.5	49.0	18.0	6.1	13.5	45.0	19.0

[1] Total red blood cell count; [2] hemoglobin, grams per 100 cc.; [3] hematocrit, volume per cent of packed blood cells.

This manuscript is a condensation of a thesis submitted to the Agricultural and Mechanical College of Texas in partial fulfillment of the degree, Master of Science in Veterinary Parasitology.

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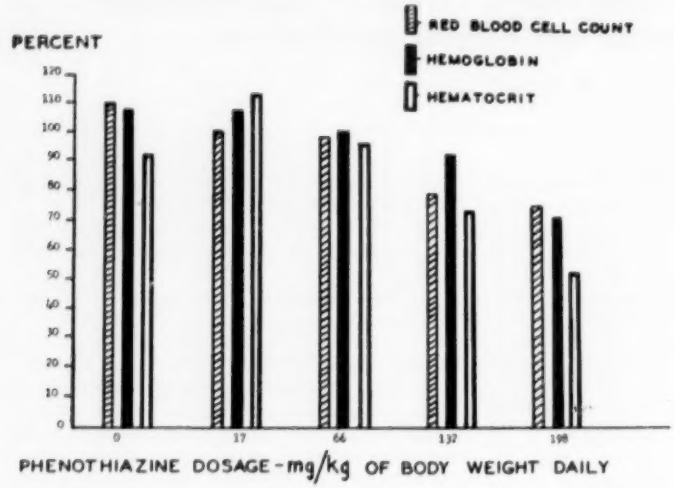


Chart 1 — Percentage of normal values of blood after giving phenothiazine to normal dogs.

dog receiving 198 mg. per kilogram of body weight became sluggish, and emaciation was evident by the fourteenth day of the trial (table 1 and chart 1).

Eighteen dogs from four litters were used to test the effect of phenothiazine on *A. caninum;* 14 of the dogs were fed phenothiazine in daily doses ranging from 4 to 33 mg. per kilogram of body weight and 4 (1 from each litter) were used as controls. These trials were divided into four experiments (table 2 and charts 2, 3, and 4).

Phenothiazine when administered in daily doses up to 66 mg. per kilogram of body weight produced no significant effect on normal healthy dogs. The erythrocyte count, hemoglobin content, and hematocrit determination of the blood did not vary significantly from normal for the individual after 60 days of medication. At the daily dose level of 132 mg. per kilogram of body weight, there was a slight decrease in blood values but it was not statistically significant. All dogs receiving up to 132 mg. per kilogram of body weight

TABLE 2 — The Effect of Phenothiazine on Dogs Artificially Infected with Ancylostoma Caninum

Dog No.	Phenothiazine mg./kg./day	Normal values				No. of larvae admin.	Day of trial ova first obser.	Values at end of experiment				No. of *A. caninum* recovered at death	Ova/Gm. at end of trial	Day of trial death occurred
		r.b.c.[1]	Hb.[2]	Hemat.[3]	Wt. (lb.)			r.b.c.	Hb.	Hemat.	Wt. (lb.)			
						EXPERIMENT 1								
6	33	5.65	14.0	39.0	9.0	1,275	0	4.28	10.5	39.0	21.0	190	0	....
7	33	5.67	14.5	43.0	8.0	1,275	0	5.5	10.5	43.0	16.0	220	0	....
8	17	6.22	14.75	42.0	13.0	1,275	48	5.54	12.0	44.0	24.0	250	850	....
9	0	6.1	15.0	45.0	15.0	1,275	15	4.67	10.0	34.0	27.0	850	37,900	....
						EXPERIMENT 2								
10	0	4.70	13.5	48.0	21.5	5,000	35	5.41	14.25	42.5	22.0	750	3,200	....
11	8	5.39	14.25	48.0	17.5	5,000	28	3.97	8.25	28.0	15.0	480	80	....
12	17	4.5	13.0	55.0	19.0	5,000	28	2.4	2.25	17.0	16.0	525	400	....
						EXPERIMENT 3								
13	17	4.25	11.0	34.0	10.0	1,800	14	1.78	3.5	12.5	9.0	480	9,709	33
14	8	3.8	9.0	28.0	11.5	1,800	13	1.95	5.75	14.0	10.0	0	450	22
15	4	4.5	9.5	38.0	12.0	1,800	14	1.29	4.0	12.0	10.0	545	3,200	24
16	0	3.4	11.4	35.0	10.5	1,800	13	0.54	2.3	8.75	7.0	1,160	1,950	18
						EXPERIMENT 4								
17	33	5.93	11.75	43.0	6.0	480	0	6.1	11.5	44.0	10.0	87	0	....
18	33	5.76	13.0	42.0	6.0	480	0	5.8	13.2	43.0	7.0	36	0	....
19	17	5.84	12.5	46.0	6.0	480	38	5.9	12.4	42.0	11.5	67	750	....
20	17	6.2	14.0	47.0	5.0	480	29	6.1	12.5	52.0	6.0	47	200	....
21	8	6.11	12.5	49.0	10.0	480	29	5.82	13.0	47.0	12.0	91	650	....
22	8	5.9	11.0	49.0	6.0	480	36	5.56	11.25	47.0	7.5	68	550	....
23	0	5.3	11.5	42.0	7.0	480	20	3.6	6.25	26.0	6.5	212	10,300	....

[1] Total red blood cell count; [2] hemoglobin, grams per 100 cc.; [3] hematocrit, volume per cent of packed blood cells.

per day appeared to be in normal health and gained in body weight. At a daily dose level of 198 mg. per kilogram of body weight, there was a marked decrease in the blood values. At this level the animals lost weight, appeared sluggish, dehydrated, and showed partial anorexia.

On necropsy, the animal receiving 198 mg. showed toxic hepatitis, nephritis, swollen lymph nodes, and cachexia.

The animals that received 8 mg. of phenothiazine daily per kilogram of body weight had a mean maximum egg-per-gram count of 432.5 while the control animals had a mean of 13,337.5. The mean percentage of larvae developing to adults was 14.2 for treated animals and 47.5 for controls. The mean percentage development of ova was 0.75 for the medicated group and 84.5 for the controls.

The animals receiving 17 mg. of phenothiazine per day per kilogram of body weight had a mean maximum ova per gram of 2,280; controls had 13,334.5. The mean percentage of larvae recovered as adults was 16.1 and 47.5 for controls. Clinical signs of ancylostomiasis were absent in the treated group but were severe in the controls.

The animals receiving 33 mg. of phenothiazine daily per kilogram of body weight did not pass ova at any time and showed no gross signs of ancylostomiasis. The mean average percentage of larvae recovered as adults was 14.4 and 47.5 for controls.

The maximum ova-per-gram count, percentage

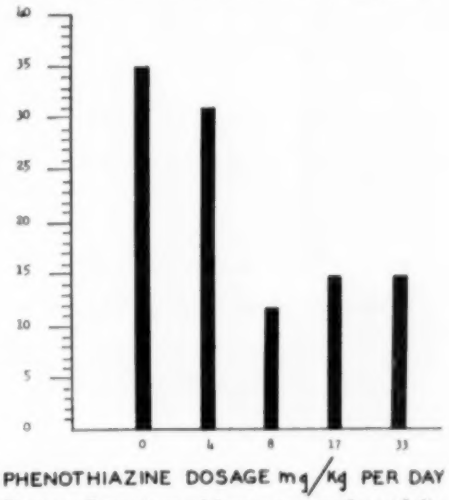
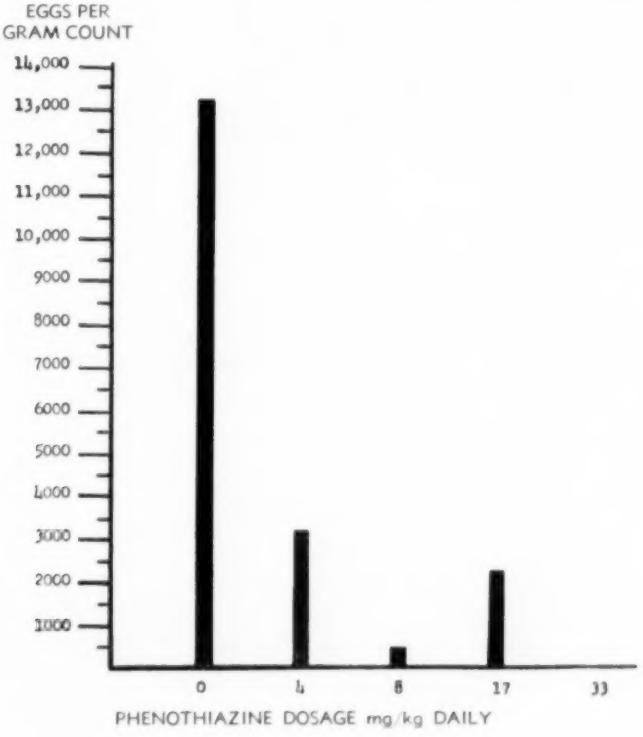


Chart 3—Percentage of larvae recovered as adults at necropsy of dogs artificially infected with Ancylostoma caninum in four experiments.

Chart 2—Average maximum egg-per-gram count after dogs were artificially infected with Ancylostoma caninum in four experiments.



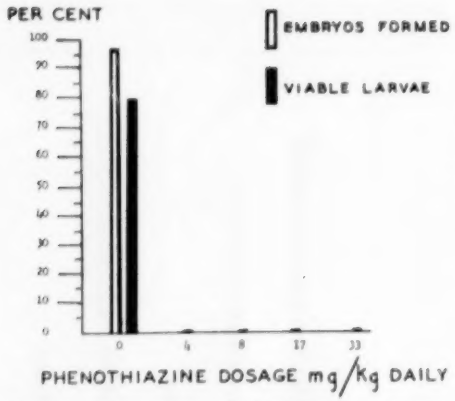


Chart 4 — Percentage of development of eggs passed by dogs artificially infected with Ancylostoma caninum in four experiments.

of larvae recovered as adults, and the percentage development of ova passed for the various groups are compared in charts 2, 3, and 4, respectively.

SUMMARY AND CONCLUSIONS

Phenothiazine in daily doses, up to 66 mg. per kilogram of body weight, for 60 days produced no recognizable signs of toxicity. Of 18 days artificially infected with *A. caninum*, 14 were fed phenothiazine daily in varying doses and 4 served as controls. The treated dogs showed a reduction in the ova per gram count of the feces, few ova that developed into viable larvae, and a reduction in the number of larvae that developed to adults.

In animals receiving 33 mg. or less of phenothiazine per kilogram of body weight there was an inverse correlation between the amount of phenothiazine given, the production of ova, and the viability of those ova.

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The Transmission of Visceral Lymphomatosis with Live Virus Newcastle Disease Vaccines

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IT HAS BEEN FOUND possible to transmit numerous diseases of the chicken from parent to offspring via the fertile egg. Cottral [6] listed nine such diseases, and others may now be added to this list.[9]

The importance of egg transmission varies greatly among infectious diseases. In general, it is of little significance in the acute diseases which run a rapid course; however, this mode of transmission becomes very important when the disease is a chronic one or when "carriers" exist that are normal in appearance and that lay a significant number of fertile eggs. Pullorum disease, chronic respiratory disease, and visceral lymphomatosis are examples of the latter type.

Since these diseases also spread readily by direct or indirect contact, their transmission is not confined to the vertical [10] (from parent to offspring), and it would appear that a vertically transmitted contagion becomes important only when it serves as a source of infection for a horizontal spread in a population of about the same age or generation.

It has been suggested [7] that another possible means of spread of egg-borne diseases is by egg-propagated vaccines. Contamination of commercial fowlpox vaccines [11, 12] and of laryngotracheitis vaccines [18] with Newcastle disease virus has been demonstrated. Such vaccines [11, 12] have also been shown to be responsible for the introduction of Newcastle disease into several chicken and turkey flocks. Thus far, no method has been developed which will eliminate or kill a contaminant in such a vaccine. If such factors as dose, route of administration of the contaminated vaccine, and the age and susceptibility of the birds are favorable, it is quite possible that a disease may be spread by this means. The contagious neoplasm, visceral lymphomatosis, may be such a disease. The presence of significant amounts of the virus causing visceral lymphomatosis in embryos of eggs laid by normal appearing hens has been adequately demonstrated by Cottral *et al.*[5] and Burmester *et al.*[4] Inoculums prepared from the liver of such embryos, when given intraperitoneally to susceptible chicks, have induced visceral lymphomatosis within nine months in over 75 per cent of chickens inoculated. In a survey of hens of two inbred lines classified as susceptible to lymphomatosis, it was found [4] that 16 of 17 hens tested shed significant amounts of virus into their eggs. In other experiments [3] a high prevalence of the disease was obtained by instilling a suspension of visceral lymphomatosis virus into the conjunctiva, external nares, cloaca, mouth, and esophagus, and by an aerosol. Several of these routes are routinely used for the administration of Newcastle disease and infectious bronchitis vaccines. Furthermore, these vaccines are used in young chickens at an age [1, 17] when they are still quite susceptible to infection with the virus of visceral lymphomatosis.

In view of these considerations, it seemed desirable to determine if lymphomatosis could be transmitted through the use of live virus Newcastle disease vaccines. After this study had been initiated, Piedrafita [16] reported on a spread of lymphomatosis which he attributed to the use of formolized Newcastle disease vaccines. It is known [4] that the virus of visceral lymphomatosis is quite sensitive to inactivation with formaldehyde and ultraviolet light. Furthermore, other modes of transmission should be considered, such as the vaccination procedure,[13] external parasites,[8] and environment.[15]

EXPERIMENTAL PROCEDURE

Chicks used in the experiments were from an inbred line of Single Comb White Leghorns (line 15) selected, at the U. S. Regional Poultry Research Laboratory, for susceptibility to lymphomatosis. This line had been propagated in isolation during the preceding 13 years for the purpose of reducing natural infection. All experimental chicks were hatched in an incubator located in a room which was isolated and under strict quarantine from any other stock. The chicks were banded at the time of hatching and transferred to battery

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brooders that were maintained under isolation. Just prior to vaccination they were divided into five lots of approximately equal numbers of each sex, and transferred to five pens in a building that was designed and constructed for the purpose of this study. It was located over 200 feet from any other building and was under quarantine at all times. Each pen was maintained as a separate isolation unit. Coveralls, coats, caps, and boots were assigned to each pen and used by the attendant in servicing each lot of birds. Disinfectant was provided for washing the hands before entering a pen.

All eggs used for the production of the specially prepared vaccines used in experiments 1 and 4 were from pedigreed Single Comb White Leghorns of the U. S. Regional Poultry Research Laboratory. The eggs were of two sorts: (1) those laid by hens of an infected flock, and (2) those laid by hens of an isolated flock. In previous tests,[4, 5] it was found that most hens of an infected flock shed the virus of visceral lymphomatosis into their eggs; whereas, no evidence of such transmission was found in two tests of eggs from the isolated flock. It can not be assumed that the latter population is entirely free from infection, since a few of the birds develop the disease annually. In the four years from 1951 to 1954, inclusive, of a total of 526 chickens maintained to 500 days of age, 1.71 per cent developed visceral lymphomatosis, and 0.73 per cent developed neural lymphomatosis.

Experiment 1.—Chicks were obtained from four biweekly hatches during the summer of 1949 and were vaccinated at 30 days of age.

Three vaccines were tested, using 242 chickens divided into five lots. Vaccine for lot 1 was prepared from half of the liver of embryos of eggs laid by hens of the infected flock, and seeded with Newcastle disease virus (NDV) after ten days of incubation. This vaccine was similar in type to the embryo liver inoculum employed by Cottral *et al.*[3] and Burmester *et al.*[4] The vaccine for lots 2 and 3 was prepared from the remaining half of the liver and half of the embryo, and from extraembryonic membranes and fluids used to prepare the liver vaccine. Three hens (J310R, J709W, and J716R) served as the specific source of eggs used in the production of these vaccines. In a previous test, inoculums prepared from embryos of these hens produced visceral lymphomatosis in 35, 33, and 47 per cent of susceptible chicks inoculated intraperitoneally.[5] The vaccine for lots 4 and 5 was prepared from the embryo, membranes, and fluids of NDV-infected embryos of eggs laid by hens of the isolated flock. The vaccines used for lots 2 and 3 and for lots 4 and 5 were similar in composition and preparation to commercial wing-web vaccines.

The seed virus used for the inoculation of the embryonating eggs was the Roakin strain* which has been used for the commercial production of live-virus vaccine to be applied by the wing-web method. Inoculations were made into the allantoic chamber of 10-day-old embryonating eggs. The dose was 0.2 ml. of a 1:100 dilution of reconstituted, dehydrated NDV-infected embryo, extraembryonic membranes and fluids. Incubation was at 99.5 F. The eggs were candled three times daily and all eggs with dead embryos were transferred to a refrigerator. Specimens were collected on the third postinoculation day from embryos that died on the second and third day.

The two vaccines were prepared in the following manner: The extraembryonic fluids were collected and the embryo deposited in a Petri dish. The liver was removed, sectioned longitudinally, and half of each liver was placed in a separate pool. The remaining parts of the embryo and extraembryonic membranes were also pooled and processed for three minutes in a chilled Waring blendor.

All collections were frozen overnight at —45 C. and then thawed at room temperature. One pool of liver was ground with a mortar and pestle with a volume of 0.85 per cent NaCl equivalent to 1 ml. for each half of liver present in the pool. This liver suspension constituted vaccine for lot 1.

The fluid and ground embryonic membrane were centrifuged at low speed for ten minutes and half of the supernatant fluid was added to the liver suspension obtained by grinding the second liver pool in a mortar with extraembryonic fluids. This mixture constituted vaccine for lots 2 and 3.

A portion of the extraembryonic fluids was diluted 1:100 and embryonating eggs of the isolated flock were inoculated in a manner similar to that already described. The embryo, extraembryonic membranes, and fluids were collected from eggs having embryos that died on the second postinoculation day. All material was pooled, ground in a chilled Waring blendor and centrifuged. The supernatant fluid was used as vaccine for lots 4 and 5.

One-milliliter portions of each vaccine in small vials were dehydrated by sublimation at 50 μ of pressure for 72 hours at room temperature. The dehydrated vaccine was stored until used at 4 C. in vacuum and under P_2O_5.

Immediately prior to use, the contents of one vial of each type of vaccine was reconstituted to volume with 1 ml. of a 25 per cent solution of glycerin in 0.85 per cent sodium chloride. The vaccines were applied by the stick or wing-web method with a conventional two-needle applicator which was dipped in the vaccine before application. Separate needles were used for each type of vaccine.

From all vaccinated lots, 27 chickens died during a three-week period following vaccination. Newcastle disease virus was recovered from 19 of the 27. Deaths which were associated with vaccination were not included in the data of the mortality which occurred during the period between 51 and 310 days of age (table 1). For ten con-

* The strains of Newcastle disease virus used in experiments 1 and 4 were supplied through the courtesy of Dr. F. R. Beaudette, New Jersey Agricultural Experiment Station, New Brunswick.

TABLE 1—The Prevalence of Lymphomatosis in Chickens Vaccinated with Newcastle Disease Virus Propagated in Eggs Laid by Hens of an Infected Flock and by Hens of a Lymphomatosis-Free Flock

Lot No.	Vaccine	Route	No. chickens	Percentage deaths: Lymphomatosis: Visceral	Percentage deaths: Lymphomatosis: Neural	Percentage deaths: Other causes
1	Infected liver only	Wingweb	47	4.3	8.5	2.1
2	Infected Com. type	Wingweb	46	15.2*	2.2	0
3	Infected Com. type	Wingweb	47	19.2*	6.0	0
4	Isolated Com. type	Wingweb	51	5.9	3.9	0
5	Isolated Com. type	Wingweb	51	2.0	3.9	0
	Nonvaccinated controls		107	1.9	7.5	0

* Differences between lots 2 and 3 and the nonvaccinated controls and between the sums of lots 2 plus 3 and lots 4 plus 5 are significant at the 1 per cent level.

secutive weeks, eggs were collected at random from each lot of chickens that had been vaccinated 19 to 25 weeks previous to the collection of the eggs. Hemagglutination-inhibition (HI) tests were conducted with yolk extracts.[16] Eggs from each of the vaccinated lots had HI titers ranging from 1800 to 4800; whereas, those from the nonvaccinated lots had HI titers ranging from 0 to 11.[16]

The data (table 1) show that neural and visceral lymphomatosis occurred in all five experimental lots. The incidence of neural lymphomatosis did not vary significantly between lots. There was only 1 per cent less of this form of the disease in the nonvaccinated controls than in the lot having the most infected birds (8.5%), thus supporting the indication that the occurrence of neural lymphomatosis was not related to vaccination. However, significant variations between lots were obtained in the incidence of visceral lymphomatosis. *Chi*-square analysis showed that differences between lots 2 and 3, which received the commercial-type vaccine, and the nonvaccinated controls were significant at the 1 per cent level. Furthermore, it was found that when the chickens of lots 4 and 5, which received the vaccine grown on eggs laid by the isolated hens, were combined and compared with the combination of lots 2 and 3, the difference in the percentage of visceral lymphomatosis was also significant at the 1 per cent level.

These results strongly indicate that live virus Newcastle disease vaccine propagated in embryonating eggs contaminated *in ovo* with the virus of visceral lymphomatosis may be capable of transmitting the disease, causing a significant increase among susceptible chickens. The results further suggest that the occurrence of neural lymphomatosis was not influenced by the administration of such a vaccine.

Experiment 2.—Chicks for this experiment, obtained from four hatches during the period of July 9 to Aug. 6, 1951, were vaccinated at 28 days of age. The two vaccines tested, A and B, in this experiment were commercial live virus Newcastle disease vaccines for administration by the wing-web route. All vaccines used from each manufacturer had the same serial number. The vaccines were applied to the wing-web, according to the manufacturer's instructions, and they were also administered intraperitoneally. For this purpose, they were diluted in 0.85 per cent saline so that 1 intraperitoneal injection of 0.25 ml. contained approximately the same amount of virus as 1 wing-web dose.

Definite reactions to the administration of these vaccines were obtained although deaths that occurred could not be directly attributed to the vaccination. Accidental flooding with water of pen 2 resulted in the loss of about half of the chicks.

The essential mortality data for chickens of this experiment, during the period of 49 to 311 days of age, are presented (table 2). Visceral lymphomatosis occurred in all five lots with a high of 20.8 per cent in lot 2, treated with vaccine A by the wing-web route, and 19.6 per cent in lot 3, inoculated intraperitoneally with vaccine B; however, 4 cases (7.7%) also occurred in the nonvaccinated control lot. Because of the occurrence of lymphomatosis in the control lot, an insignificant *chi*-square in testing for homogeneity was obtained, thus indicating that the various lots could have been drawn from a single population.

Experiment 3.—The chicks were hatched on Oct.

TABLE 2—The Prevalence of Lymphomatosis in Chickens Vaccinated with Two Commercial Newcastle Disease Vaccines

Lot No.	Vaccine	Route	No. chickens	Percentage deaths: Lymphomatosis: Visceral	Percentage deaths: Lymphomatosis: Neural	Percentage deaths: Other deaths
1	Com. live virus A	Intraperitoneal	45	6.7	6.7	0
2	Com. live virus A	Wingweb	24	20.8*	0	12.5
3	Com. live virus B	Intraperitoneal	46	19.6	0	0
4	Com. live virus B	Wingweb	42	11.9	7.2	0
5	Nonvaccinated controls		52	7.7	0	0

* Differences between lots were not significant by the *chi*-square test.

20, 1952, and at 2 days of age were divided into five lots and vaccinated. Commercial vaccines from four different manufacturers were tested in this experiment. Three of these vaccines (C, D, and E) were of the live virus wing-web type. They were administered by intramuscular injection of 0.1 ml. of a dilution of vaccine containing 1 wing-web dose. The fourth vaccine (F) was a commercial killed virus Newcastle disease vaccine and was administered by intramuscular injection of 0.25 ml. of undiluted vaccine.

Vaccine C produced only a moderate response in the baby chicks, resulting in a low mortality during the first three weeks of life. However, vaccines D and E produced a more severe reaction, which resulted in the loss of about half of the experimental chicks. This occurred during the first three weeks and signs of, or deaths due to, Newcastle disease were not seen after this period.

Mortality among the birds that survived vaccination, and which occurred during the period of 30 to 298 days of life, has been summarized (table 3). A relatively high prevalence of visceral lymphomatosis occurred in one of the three lots of chickens which had received live virus vaccines. The differences between lot 2, which received vaccine D, and the nonvaccinated controls in percentage of cases of visceral lymphomatosis was significant at the 1 per cent level. Four cases also occurred in the lot which had received the live virus vaccine E but none occurred in lot 1 (vaccine C) and none in the killed virus vaccinated group. Despite the lack of transmission with vaccine C and the low rate of infection in the chickens that received vaccine E, the significant results obtained with vaccine D of this experiment suggest that commercial vaccines may contain sufficient virus of visceral lymphomatosis to induce a significant increase in this disease.

An appreciable number of cases of neural lymphomatosis also occurred in the various lots of this experiment; however, the variation between lots was not significant, indicating that factors other than the vaccines and common to all lots were responsible for the appearance of neural lymphomatosis.

Experiment 4.—Chicks hatched Sept. 10, 1953, were vaccinated at three weeks of age by an intramuscular injection of 0.2 ml. of specially prepared vaccines. The LaSota strain (N.J. 717) of Newcastle disease virus was used for this experiment. This strain had been employed previously for vaccination by intramuscular injection of a live virus preparation in contrast to the wing-web type strains employed in the previous three experiments. The intramuscular-type strain was grown in eggs from individual hens which in previous tests were shown to pass the virus of visceral lymphomatosis in their eggs. The inoculation of these eggs, the harvesting and the preparation of the vaccines were done in a manner similar to that described for the vaccines used in experiment 1, except that the entire embryo, extraembryonic membranes, and fluids of eggs from 1 hen were pooled and processed in a chilled Waring blendor. Just prior to vaccination, each reconstituted vaccine was diluted 1:3 with 0.85 per cent saline.

The vaccine for lot 1 was produced in 11 embryonating eggs laid by hen N720S, vaccine for lot 2 was grown in 14 eggs laid by hen N436N, and that used in lot 3 was propagated in 12 eggs of hen N423W. These eggs were laid during the month of August. Samples of embryonating eggs laid during the months of April, July, and October were tested for the presence of the virus of visceral lymphomatosis by the intraperitoneal injection of day-old chicks with inoculums prepared from the livers of 15-day-old embryos of the eggs under test. They were found to contain sufficient virus to produce a high incidence of tumors in all the tests conducted except for the egg samples laid in July by N436N and N423W. The vaccine for lot 4 was grown in 30 embryonating eggs selected at random from those laid by hens of the isolated line-15 population. Tests for the presence of the virus of visceral lymphomatosis in eggs of 2 hens of this population gave negative results.

Signs of Newcastle disease, which could be directly attributed to vaccination, were observed. No deaths occurred during a three-week, postvaccination period.

The mortality which occurred during the period of 42 to 350 days of age for birds of this experiment is summarized (table 4). All cases of lymphomatosis occurred in two lots that had received vaccines prepared from infected eggs. In lot 1, 21.7 per cent of 69 birds developed visceral lymphomatosis and 3 birds of lot 3 died of this disease. No cases developed in the two control lots 4 and 5, and in lot 2 which received vaccine from eggs of 1 of the infected hens. No cases of neural lymphomatosis developed. Many birds showed nervous symptoms commonly observed in birds

TABLE 3—The Prevalence of Lymphomatosis in Chickens Vaccinated with Four Commercial Newcastle Disease Vaccines

Lot No.	Vaccine	Route	No. chickens	Percentage deaths		
				Lymphomatosis		Other causes
				Visceral	Neural	
1	Com. live virus C	Intramuscular	41	0	2.4	2.4
2	Com. live virus D	Intramuscular	19	31.6*	15.8	31.6
3	Com. live virus E	Intramuscular	27	14.6	22.2	25.9
4	Com. killed virus F	Intramuscular	59	0	1.7	8.5
5	Nonvaccinated controls		53	1.9	7.5	5.7

* Significant at 1 per cent level.

having Newcastle disease. They became emaciated and died and were largely responsible for the high percentage of mortality listed in the table as "other causes."

The results indicate that one of the vaccines tested in this experiment contained enough of the virus of visceral lymphomatosis to produce a significant number of cases of the disease. It would further appear that the intramuscular route was efficacious in transmitting the disease.

DISCUSSION

Four experiments involving 1,103 chickens were conducted in sequence over a period of five years for the purpose of studying the possibilities of transmitting visceral lymphomatosis by live virus Newcastle disease vaccines. In three of these four experiments, positive evidence for such transmission was obtained. In experiment 1, a NDV strain employed for vaccination by wing-web application, and in experiment 4 a strain employed for vaccination by intramuscular injection, were propagated in embryonating eggs laid by hens of the U. S. Regional Poultry Research Laboratory which had been shown to be shedders of the virus of visceral lymphomatosis. Vaccines prepared from such eggs in the usual manner and administered by the wing-web method, with a conventional two-needle applicator, and by intramuscular injection, resulted in percentages of visceral lymphomatosis which were significantly (at 1% level of probability) greater than that obtained in control lots. Some of the lots that had received vaccines prepared from eggs laid by hens that were known to have shed the virus did not show a significant incidence of this disease. These inconsistent results are not readily explainable. In the case of the fourth experiment where the eggs of individual hens were used, variation in the amount of the virus shed at different periods may be a factor. Evidence for such a phenomenon has been obtained in other studies.[19] Evidence was obtained also for a low rate or absence of shedding during a period just prior to the laying of eggs by the 2 hens used for preparation of vaccine for the two lots that gave negative transmission results.

In experiments 2 and 3, commercial live virus Newcastle disease vaccines were administered by the wing-web method and by intraperitoneal and intramuscular injections. One of three vaccines used in experiment 3 caused a highly significant incidence of visceral lymphamotosis. In experiment 2, differences between the nonvaccinated control lot and four other lots that had received two commercial vaccines each, by two routes of administration, were not significant. The prevalence of the disease in the control lot of this experiment was much higher than that of the control lots of the other three experiments. This may have been due either to an unusual amount of egg-borne infection or to a break in the isolation between pens which allowed the spread of infection from the vaccinated lots to the control lot.

Although transmission of visceral lymphomatosis was not obtained at a high rate nor in all vaccinated lots, sufficient positive data have been obtained to indicate that this disease may be transmitted by live virus Newcastle disease vaccines. In one test, a commercial vaccine was incriminated and two experimental vaccines produced transmission. These results were obtained with chickens of an inbred line that had been selected for over 13 years for their susceptibility to lymphomatosis. Furthermore, they had been maintained for most of this period in isolation for the purpose of minimizing exposure to this disease. These chickens have been highly susceptible to visceral lymphomatosis, not only to inoculation with the filtrable agent

TABLE 4—The Prevalence of Lymphomatosis in Chickens Vaccinated with Newcastle Disease Virus Propagated in Eggs Laid by Hens of an Infected Flock and by Hens of a Lymphomatosis-Free Flock

Lot No.	Vaccine	Route	No. chickens	Percentage deaths: Lymphomatosis: Visceral	Lymphomatosis: Neural	Other causes
1	Infected Com. type (N720S)	Intramuscular	69	21.7*	0	24.6
2	Infected Com. type (N436N)	Intramuscular	70	0	0	22.8
3	Infected Com. type (N423N)	Intramuscular	67	4.5	0	13.4
4	Isolated flock	Intramuscular	70	0	0	25.7
5	Nonvaccinated controls		70	0	0	15.7

* Significant at 1 per cent level.

but by natural means of exposure.[3] The basis for this susceptibility, no doubt, is largely genetic; however, it is probable that other factors, such as the lack of prior infection with the visceral lymphomatosis agent, also may be important.

Neural lymphomatosis occurred in all lots of two of the four experiments and was almost absent in the other two experiments. The apparent variation in neural lymphomatosis between experiments is not readily explainable. For each experiment the same building and equipment were used. The same caretaker and isolation procedures were employed in all experiments. The age at vaccination and moving from batteries to the five-pen experimental unit was four weeks for experiments 1 and 2, three weeks for experiment 5, and two days for experiment 4. Thus, there was no particular relationship between age at vaccination and the occurrence of neural lymphomatosis. Neither was there any apparent relationship between routes of administration and the prevalence of neural lymphomatosis.

Because of a lack of significant differences between any of the lots in the two experiments in which neural lymphomatosis occurred, it would appear that this disease was not related to vaccination or to the vaccines employed but rather to some unknown factor or factors common to all lots but variable between experiments. The finding that the occurrence of neural lymphomatosis was not influenced by administration of live virus, egg-propagated vaccines, whereas visceral lymphomatosis was so influenced, was to be expected on the basis of findings reported by Cottral *et al.*[8] and Burmester *et al.*[4] These investigators repeatedly were able to produce visceral lymphomatosis with inoculums prepared from "normal" 15-day-old chicken embryos but neural lymphomatosis either occurred not at all or at a very low rate. These results provide evidence that neural and visceral lymphomatosis have a separate etiology.

SUMMARY

1) Four experiments, involving 1,103 chickens distributed among 21 lots, were conducted in studies to ascertain the possibility of transmitting visceral lymphomatosis by live virus Newcastle disease vaccines.

2) In two experiments, chickens that had received vaccines prepared from embryonating eggs laid by hens that shed the visceral lymphomatosis virus had a significantly greater prevalence of visceral lymphomatosis than nonvaccinated controls or chickens receiving vaccine prepared from eggs laid by hens of an isolated population that had been shown not to shed the virus.

3) In two other experiments in which commercial vaccines were tested, the prevalence of visceral lymphomatosis in one of the vaccinated lots was significantly higher than that in the nonvaccinated controls.

4) The data indicate that ovarian transmission of visceral lymphomatosis virus may result in sufficient contamination of live virus Newcastle disease vaccines to result in a significant transmission of visceral lymphomatosis in highly susceptible stock. Neural lymphomatosis was not transmitted by live virus Newcastle disease vaccines under the conditions in which the transmission of visceral lymphomatosis occurred.

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Preparation of a New Pleuropneumonia-like Organism Antigen for the Diagnosis of Chronic Respiratory Disease by the Agglutination Test

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THE agglutination test was shown by Adler [1] and by Jacobs *et al.*[2] to be of value in the detection of agglutinins in the blood of chickens and of turkeys infected with pleuropneumonia-like organisms (PPLO). These authors have previously reported a satisfactory agglutination test antigen. Other research workers, however, have been unable to duplicate these published results, apparently due to the difficulty in attaining a satisfactory PPLO growth for antigen production. It is the purpose of this paper to present the development of cultural methods for production of a satisfactory PPLO antigen to detect chronic respiratory disease of chickens (CRD) and infectious sinusitis of turkeys.

EXPERIMENTAL DATA

Growth Cycle of PPLO of Avian Origin.—It appeared desirable to determine the phases of growth of avian PPLO in artificial mediums and in chicken embryos based on actual colony counts rather than by lethal dose ($l.d._{50}$) as previously reported.[3, 10] While the results would indicate trends rather than statistically finite points, it was felt that the data would furnish information applicable to later research.

In studies of the growth cycle, a PPLO designated as strain C which was isolated from a CRD case and had been propagated through 33 chicken embryo passages was used. Mediums used throughout the study were PPLO agar and broth (Difco) enriched with 20 per cent horse serum.

Yolk material from embryo passage 33 of strain C was inoculated into the yolk sacs of 40 six-day-old chicken embryos according to the method of Cunningham.[4] Additionally, 1 ml. of the same inoculum was inoculated into 50 ml. of horse serum-enriched broth. At intervals, portions were taken from the yolk sacs of living embryos and from the broth culture and serially diluted in heart infusion broth (Difco). One-tenth milliliter of each dilution was seeded into each of five tubes containing 0.9 ml. of a 20 per cent horse serum-enriched broth.

After three days of incubation at 37 C., the tubes of broth were plated with a 5-mm. loop on 20 per cent serum-enriched agar plates. Three days later the plates were examined with a dissecting microscope at a magnification of 36 times for the presence or absence of growth. Questionable colonies were stained by the method of Dienes [5] and examined at 500 and 960 diameters. The probable number of organisms was determined from tables published by McCrady as modified by Buchanan and Fulmer.[3]

After repeating the trials for confirmation of findings, growth curves of the PPLO were computed (fig. 1). It is apparent that the growth in embryos did not manifest a definite lag phase as is evident in culture mediums. The difference is possibly due to adaptation of the organism to embryos as a result of previous 33 chicken embryo passages.

Growth of Avian PPLO in Mediums Enriched with Yeast Products.—Edward [6] demonstrated that an aqueous extract of boiled brewer's yeast contained a factor which enhanced the growth of PPLO in horse serum-enriched broth. Unpublished work by the authors of this paper suggested that yeast products, when added to serum, stimulated the growth of the avian PPLO. To verify this observation, four yeast products were prepared: (1) A yeast autolysate was prepared using one part of baker's yeast and one part of distilled water. This mixture was preserved with a thin layer of a volatile preservative * and autolysed at 55 C. for three days and then centrifuged at 2,000 r.p.m. for 30 minutes. The supernatant was filtered through a Buchner funnel packed with a diatomaceous silica filter aid.** The filtrate was adjusted to pH 7.8 with 1 N NaOH. Finally the yeast autolysate was either autoclaved (121 C. for 10 minutes) or autoclaved and the resulting fluid passed through a seitz filter. (2) A yeast hydrolysate was prepared using two parts of baker's yeast and one part of distilled water. This mixture was autoclaved at 121 C. for 15 minutes after which the volume of the water was increased to give a final proportion of one part of yeast to one part of water. The same procedures were then followed as described for the yeast autolysate for the purification process. (3) A commercial yeast autolysate (Difco) was reconstituted to a 5 per cent solution in PPLO broth (Difco) and filtered to remove the particulate matter. The filtrate was adjusted to pH 7.8 with 1 N NaOH and then autoclaved at 121 C. for 15 minutes. (4) Commer-

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* A mixture of chlorobenzene, 1-2 ethylene dichloride, and 1-chlorobutane.

** A Johns-Manville product, Standard Super-Cel.

cial yeast extract (Difco) was added directly to PPLO broth in concentrations of 2.5, 5.0, and 10.0 per cent, respectively, the mediums were adjusted to pH 7.8 and autoclaved at 121 C. for 15 minutes.

Except for the fourth preparation, these yeast products were added to a basal PPLO broth (Difco) without crystal violet on a volume basis. The inoculum for the study was a 24-hour culture of the eighty-fifth passage of a PPLO isolated by Zander [12] from the brain of a turkey which had exhibited encephalitic symptoms. The variously enriched broth mediums were inoculated with the PPLO culture equal to 1 per cent of the final volume. Cultures were incubated at 37 C. for ten days. After five days of incubation, samples were plated on 20 per cent serum agar to test for both contamination and evidence of PPLO growth. After ten days of incubation, determination of the growth of PPLO was accomplished by centrifugation of 45-ml. aliquots of the broth cultures at 10,200 g for 30 minutes in a Serval angle centrifuge (type SS-1). The sediment was resuspended in 10 ml. of buffered saline at pH 6.8. Turbidity was measured by transmittance by using the Klett-Summerson photoelectric colorimeter. To determine that the sediment represented PPLO and not merely debris, the samples were recentrifuged, and 2 ml. of antigen was prepared from each. The slide agglutination test was then conducted against known positive PPLO serums in a similar manner as described by Adler.[1] The effects of these yeast products at varying concentrations on the growth of PPLO as measured by colorimetry and by the agglutination reaction are shown (table 1). These tests were repeated with similar results.

Supplementary data were acquired from a study of four other strains of PPLO isolated from avian sources using the medium enriched with yeast autolysate, the yeast product which had demonstrated the most satisfactory results in the growth study with the Zander strain. Three of the strains of PPLO had been isolated from tracheal exudates of chickens with CRD and the other was isolated from the sinus exudate of a turkey with infectious sinusitis. These four strains were cultured in a repetition of the procedure presented in the previous paragraph, with results similar to that obtained with the Zander strain.

Studies were next undertaken to determine the optimum serum and yeast autolysate concentrations required to give maximum growth of the Zander strain PPLO. Doubling dilutions of yeast autolysate and horse serum were made in PPLO broth and in PPLO agar resulting in mediums of 0, 2, 4, 8, and 16 per cent yeast and the same of serum. The variously enriched broth mediums were inoculated with the Zander strain PPLO, incubated for ten days at 38 C., and harvested by centrifugation of 45-ml. aliquots at 10,200 g for 30 minutes. The sediment was resuspended in 10 ml. of Sorensen's phosphate buffered saline (pH 6.8). The density of growth was measured by the colorimeter as described previously. The results are illustrated in figure 2.

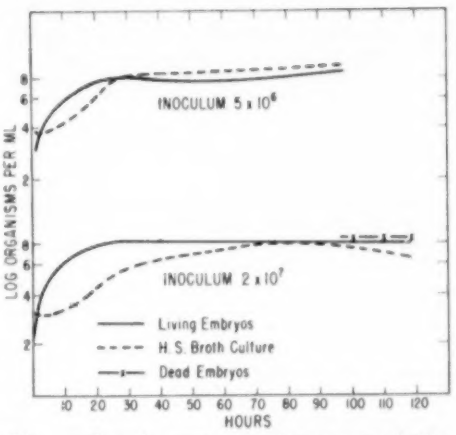

Fig. 1—Growth curve of pleuropneumonia-like organisms (PPLO) in broth and in the yolk sac of embryonating chicken eggs. One milliliter of PPLO-infected yolk was seeded into 50 ml. of broth. The chicken embryos were inoculated into the yolk sac with 0.1 ml. of the PPLO yolk.

For the study of PPLO growth on agar containing the varying concentrations of yeast and serum, a three-day broth culture of the Zander strain PPLO was propagated in 20 per cent horse serum broth for 72 hours. The growth was harvested by centrifugation at 14,000 g for 20 minutes in a refrigerated centrifuge. The sediment was washed twice in PPLO broth containing neither serum nor yeast to free the organisms of any enrichments from the culture medium and was recentrifuged at the same speed. A small drop of this washed-cell sediment was seeded onto each agar plate of different concentrations of serum and yeast enrichments, incubated at 38 C. for 72 hours, and then examined under the dissection mi-

TABLE 1—Effect of Four Yeast Supplements on the Yield of Pleuropneumonia-like Organisms when Propagated for Ten Days in 8 per cent Horse Serum-Enriched Broth

Yeast supplement	Klett reading * (turbidity)	Agglutination **
2.5% Difco yeast extract	44	Direct+
5.0% Difco yeast extract	78	1:2+
10.0% Difco yeast extract	31	Direct—
8.0% horse serum	68	1:2+
16.0% yeast hydrolysate	118	1:2++
16.0% yeast autolysate	136	1:2+++
16.0% Difco yeast autolysate of a 5% solution	93	1:2+

* The greater the reading, the more turbid the sample; ** all antigens were prepared from 45 ml. of culture centrifuged and resuspended to 2.0 ml. in buffered saline; direct+ poor agglutination of undiluted antigen; direct— no agglutination of undiluted antigen; 1:2+ poor visible agglutination of 1:2 dilution of antigen; 1:2++ moderate visible agglutination of 1:2 dilution of antigen; 1:2+++ good visible agglutination of 1:2 dilution of antigen.

croscope. The growth response was based on the size and the number of PPLO colonies. The trend of growth, in general, followed the results obtained in broth, but it was not possible to assign absolute values to the different degrees of growth on agar mediums.

Enhancement of Avian PPLO Growth by Agitation.—Recently it was shown by Jacobs *et al.*[6] that stirring of the PPLO culture during incubation increased the yield of organisms. They used an electrically driven magnetic stirrer and incubated the culture at room temperature. Experience of the authors of this paper with this method showed that the yield of organisms was not constant, probably due to variations of temperature of the room and heat given off by the stirrer. Under conditions of room temperature inside a draft-free cubicle the temperature of the broth due to friction of the electric motor and stirrer would reach approximately 38 C., varying with the size of the container, the amount of broth, and other physical conditions.

To overcome this difficulty, an air-driven magnetic stirrer was used for agitation of the medium. The apparatus and the inoculated broth flask were placed in an incubator in order to maintain a constant temperature of 38 C. This method of agitation was the only method evaluated in this study. A thorough review of the subject was recently presented by Finn.[7]

In the evaluation of the effects of agitation on PPLO growth, 10 ml. of a 24-hour culture of the eighty-fifth passage of the Zander strain PPLO were seeded into each of two 1-liter Erlenmeyer flasks containing PPLO broth enriched with 8 and 16 per cent of serum and yeast autolysate, respectively. The surface for exchange of gases was approximately 6 cm. in diameter. One sample was agitated with the air-driven stirrer, and the other was not stirred. Both were incubated at 38 C. for ten days and, then, aliquots were harvested by the procedure described previously. The Klett reading for the stirred sample was 138 as compared to 92 for the unstirred sample, which indicated that stirring enhanced the growth and final yield of PPLO.

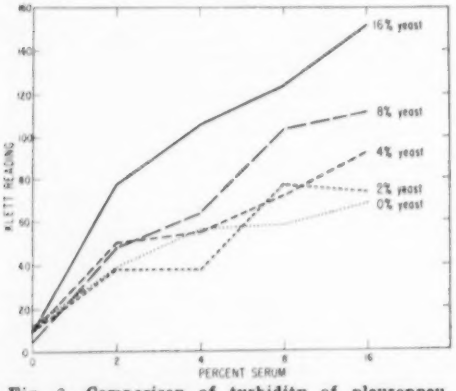

Fig. 2—Comparison of turbidity of pleuropneumonia-like organisms propagated for ten days in broth supplemented with various concentrations of yeast autolysate and horse serum.

In order to determine whether the difference in growth of PPLO due to agitation could be attained by merely increasing the exposed surface area, 5 liters of PPLO broth enriched with 8 and 16 per cent horse serum and yeast autolysate, respectively, were placed in each of two 12-liter boiling flasks to a depth of 11 cm., each with an exposed surface diameter of approximately 30 cm. Each lot was seeded with equal amounts of the Zander strain PPLO and both were incubated for ten days, one being constantly stirred, the other not. The turbidity of the harvest as measured in Klett units was 112 for the stirred sample and 104 for the motionless culture. The slight difference in readings obtained with the two samples was not considered significant.

Concentration of the Antigen.—The concentration of PPLO from a large volume of broth culture has posed an important problem. Newing and Field [11] used a cream separator to concentrate bovine pleuropneumonia organisms from broth. The writers of this paper obtained a Montgomery Ward electric cream separator (model 35HM-s-30M-A) having a speed of approximately 10,500 r.p.m. A PPLO culture from a previous experiment was passed twice through the separator at approximately 75 ml. per minute. The organisms which were deposited inside the bowl shell were washed off with buffered saline. An equal amount of the same culture was spun in an angle head, high speed Serval centrifuge at 10,200 g for 30 minutes in order to compare the yield of organisms harvested by the two methods. From the 250 ml. of broth culture used by each method, 20 ml. of antigen were prepared and turbidity measured by the colorimeter. The Klett readings were 255 for the centrifuged sample and 236 for the separator-harvested sample. These readings would seem to indicate very little differences in the yield by the two methods of harvesting.

Antigen Production.—A PPLO antigen was prepared utilizing 16 per cent yeast autolysate and 8 per cent horse serum supplemented in PPLO broth (Difco). The cultures were propagated in 5-liter amounts in 12-liter boiling flasks with constant stirring. Samples taken at intervals indicated that a uniformly high yield of antigen was obtained after ten days of incubation. Approximately 500 ml. of antigen standardized to two times the density of tube ten of the McFarland nephelometer were obtained from 10 liters of culture when harvested with the milk separator. All antigens were suspended in a phosphate buffered saline (pH 6.8). Crystal violet and aqueous merthiolate were added to a final concentration of 1:10,000 each. Phenol at a final concentration of 0.25 per cent when substituted for the merthiolate was also satisfactory. Each milliliter of antigen provided enough material for over 100 agglutination tests.

Originally the antigens were preserved with 1:25,000 crystal violet and brilliant green,[1] but it was found that these preservatives did not prevent bacterial growth under conditions of handling in the California State Diagnostic Laboratories. Furthermore, the high NaCl concentration of 10 per cent used as a suspending medium in the preparation of the antigens in the earlier studies[1] was not required with the Zander strain of PPLO used in this study. Apparently the agglutination sensitivity of PPLO of avian origin varies, depending on the strain encountered, some requiring high salt concentrations to bring about agglutination with positive serums, while others are sensitive at lower salt concentrations. The problem of NaCl concentration in avian PPLO antigen preparation has been discussed in greater detail by Adler.[2]

Antigens were prepared with four other PPLO of avian origin by the above method. All four strains grew readily and gave equal yields as the Zander strain. Two of these strains failed to agglutinate known positive serums from infected but not hyperimmunized birds, while the other two were satisfactory for antigen production. Whether the two strains which did not agglutinate positive serums would do so at higher salt concentrations was not determined.

SUMMARY

The growth curve of PPLO of avian origin in broth and in chicken embryos has been described, with evidence that the peak of growth is reached in 36 hours.

The effect on the growth of PPLO of horse serum-enriched mediums supplemented additionally with four different yeast preparations has been determined. The results indicated that yeast autolysate was the most satisfactory yeast product when added to horse serum broth. This combination of supplements when added to PPLO broth significantly increased the yield of antigen as compared to that obtained in the basal broth supplemented with serum only.

A satisfactory method of agitation of PPLO broth cultures was outlined. The growth of the organisms was shown to be enhanced by stirring during incubation, especially when cultures were propagated in flasks which provided a small surface area of broth. When surface areas of broth were large, stirring resulted in no marked increase in yield.

A cream separator has been found to be satisfactory for the concentration of large amounts of broth cultures.

A simple, efficient method of production of a PPLO antigen giving high yields has been given.

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Interference Between Newcastle Disease and Infectious Bronchitis Viruses

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SIMULTANEOUS exposure of an animal host with two viruses may result in a mixed infection, viral interference, or viral exaltation. Dual viral infections may occur when a single cell or different cells are invaded.

The invasion of a single cell by two viruses has been demonstrated by the presence of different viral inclusions within a single cell.[1-3] Interference has been demonstrated between many viruses.[3] The exact mechanism of viral interference is unknown; however, current opinion favors some interfering mechanism at a cellular level.[3-5] Exaltation of one virus by another also has been observed occasionally. Findlay and Howard[6] noticed an increased virulence of Columbia SK virus for mice when inoculated along with mouse-adapted poliomyelitis virus. Lepine and Marcenac[7] found mice infected with herpes virus were more susceptible to Aujesky's virus.

Simultaneous exposure of chickens to Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) may cause either a dual infection or an interference of the NDV by the IBV.[8] Hitchner, White, and Lozano[9] reported a reduction in HI titers when IBV and NDV were administered in a combined vaccine. Luginbuhl[10] reported that IBV and NDV will grow simultaneously in chicken embryos if both viruses are inoculated in high concentration but found decreasing growth of NDV if the inoculating fluid contained diluted NDV in the presence of undiluted IBV. Groupé[11] demonstrated interference between inactive and active IBV particles in chicken embryo studies.

Henle[3] stated that variation in viral concentrations had a very important effect upon the results obtained from simultaneous exposure to two viruses. In our previous mixed infection studies, we had observed that freezing and thawing of NDV reduced the interference by IBV (unpublished). According to Hardy and Horsfall[12] normal allantoic fluid contains a component which interferes with the ability of influenza virus to agglutinate red blood cells. Some viruses have the ability to produce an interfering effect for periods of a few days to several weeks when given separately.[3] Tsujimoto[13] found that a 72-hour preinoculation of chicken embryos with Japanese B encephalitis virus resulted in reduced ND virus propagation.

The factors studied in this experiment were virus concentration, effect of freezing and thawing, effects of diluents, and time of exposure, as related to the interference between NDV and IBV.

MATERIALS AND METHODS

The chicks used in the following experiments were White Plymouth Rocks obtained at 1 day of age. Each group of chicks was held for four weeks to allow diminution of any parental immunity which might have been transmitted through the yolk. Composite serum samples were obtained by bleeding 5 chicks from each group at 4 weeks of age and pooling equal quantities of serum. The serum samples were tested for the presence of ND and IB antibodies.

At 4 weeks of age, groups of 25 chicks each were placed in separate houses which were attended by separate caretakers. After the virus inoculations were made, symptoms and mortalities were recorded daily for two to three weeks. At the conclusion of the observation, blood was taken from 5 chicks from each lot and the serums were tested for the presence of ND and IB antibodies. The serum samples were pooled and examined in each lot in experiments 1, 2, and 4 and examined separately in experiment 3. The *alpha* hemagglutination-inhibition (HI) test was used to measure ND antibody titer, and a serum-neutralization test[14] was used to evaluate the IB antibody level. *Alpha* HI titers of 1:80 or more were considered positive for Newcastle disease. An embryo neutralization index of 10^2 or greater was considered positive for infectious bronchitis.

The viruses used were California strain 11914 of NDV and Illinois strain 70768 of IBV. The viruses were propagated in 10-day-old chicken embryos at 37 C. for 36 to 48 hours and then cooled at 4 C. for three to six hours before harvesting. The allantoamniotic (a.a.) fluids were harvested from both live and dead embryos. The NDV killed approximately 50 per cent of inoculated embryos ($e.l.d._{50}$ 10^5) at a dilution of 10^{-8} and the IBV had an $e.l.d._{50}$ of approximately 5×10^4.

All chick inoculations were made intranasally with approximately 0.05 ml. of virus. The viruses were held for 12 to 24 hours at 4 C. until used, except in experiment 4 when the NDV was kept frozen at −20 C. for one month. The NDV and IBV for the dual exposures were combined in equal portions approximately one hour before they

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were used for inoculation. The vehicles used for diluting the NDV were saline, nutrient broth, and a.a. fluid from normal 14-day-old chicken embryos. Different diluting fluids were used to observe their effect, if any, on the virus.

RESULTS

Experiment 1.—In this experiment, the NDV was used as undiluted a.a. fluid and as a.a. fluid diluted 10^{-5} and 10^{-7} in nutrient broth. The IBV was used as undiluted a.a. fluid. The chicks were divided into seven groups of 25 birds each at 4 weeks of age. The groups received the following virus exposures: group 1, NDV undiluted; group 2, NDV undiluted and IBV undiluted; group 3, NDV diluted 10^{-5}; group 4, NDV diluted 10^{-5} and IBV undiluted; group 5, NDV diluted 10^{-7}; group 6, NDV diluted 10^{-7} and IBV undiluted; and group 7, IBV undiluted. Respiratory symptoms were observed in all groups except 5, which received NDV diluted 10^{-7}. Nervous symptoms and deaths were observed in groups 1, 2, and 3. The symptoms observed and the losses in each group during the 16-day period are shown (table 1).

Group 2, with a loss of 28 per cent, received the same concentration of NDV as group 1, which had a loss of 56 per cent, thus indicating interference of NDV by the IBV. In group 4, interference of NDV also occurred as evidenced by no deaths, while group 3, which received the same concentration of NDV, had a loss of 16 per cent. Group 5, which received NDV diluted 10^{-7}, showed no evidence of respiratory or nervous symptoms and was found susceptible when subsequently challenged with undiluted NDV.

Experiment 2.—This experiment was similar to experiment 1 except two diluting fluids, saline, and normal a.a. fluid, were used and the 10^{-7} dilution of NDV was omitted. Normal a.a. fluid from 14-day-old embryos was used to determine if some factor was present in the a.a. fluid containing the viruses which might help produce the interference of NDV by IBV. Saline was used as a comparison diluent instead of nutrient broth in order to eliminate possible interference by protein molecules.

The different groups received the following exposures: group 1, NDV undiluted; group 2, NDV undiluted and IBV undiluted; group 3, NDV diluted 10^{-5} in saline; group 4, NDV diluted 10^{-5} in saline and IBV undiluted; group 5, NDV diluted 10^{-5} in normal a.a. fluid; group 6, NDV diluted 10^{-5} in normal a.a. fluid and IBV undiluted; and group 7, IBV undiluted. The total losses during the 21-day period after inoculation are listed (table 2).

Respiratory symptoms were observed in all groups. Nervous symptoms were observed in all groups except 7, which received IBV alone. Interference of NDV by IBV was evident in group 2, which had a loss of only 24 per cent, compared with group 1, which had a 56 per cent loss with a comparable NDV exposure. Interference was also evident in group 6, which had a loss of 8 per cent, compared with group 5, with a loss of 40 per cent. The high losses in group 5 indicated that the normal a.a. fluid contained no interfering factor. Losses were very low in group 3, suggesting that the saline used as a diluent had a deleterious effect on the NDV.

TABLE 1—Results of Chick Inoculation Studies in Experiment 1

Group	Inoculum	No. of chicks	Symptoms	Losses			HI Titer
				Mortality	CNS dist.*	Total loss** (%)	
1	NDV undiluted	25	R and CNS	10	4	56	+
2	NDV undiluted IBV undiluted	25	R and CNS	7	0	28	+
3	NDV diluted 10^{-5}	25	R and CNS	3	1	16	+
4	NDV diluted 10^{-5} IBV undiluted	25	R	0	0	0	—
5	NDV diluted 10^{-7}	25	R	0	0	0	—
6	NDV diluted 10^{-7} IBV undiluted	25	R	0	0	0	—
7	IBV undiluted	25	R	0	0	0	—

NDV—Newcastle disease virus; IBV—infectious bronchitis virus; R—respiratory symptoms; CNS—central nervous system disturbances; HI—hemagglutination inhibition.

* Chicks with central nervous disturbances evident at end of observation period; ** total loss includes mortality and CNS disturbance.

TABLE 2—Results of Chick Inoculation Studies in Experiment 2

Group	Inoculum	No. of chicks	Symptoms	Losses			Positive HI titers	IB SN titers
				Mortality	CNS dist.*	Total loss** (%)		
1	NDV undiluted	25	R and CNS	12	2	56	5/5	—
2	NDV undiluted IBV undiluted	25	R and CNS	6	0	24	5/5	+
3	NDV 10^{-5} in saline	25	R and CNS	1	0	4	0/5	—
4	NDV 10^{-5} in saline IBV undiluted	25	R and CNS	0	1	4	0/5	+
5	NDV 10^{-5} in a.a. fluid	25	R and CNS	9	1	40	3/5	—
6	NDV 10^{-5} in a.a. fluid IBV undiluted	25	R and CNS	2	0	8	4/5	+
7	IBV undiluted	25	R	3†	0	12	0/5	+

NDV—Newcastle disease virus; IBV—infectious bronchitis virus; R—respiratory symptoms; CNS—central nervous system disturbances; a.a.—allantoamniotic; SN—serum neutralization.

* Chicks with central nervous disturbances evident at end of observation period; ** total loss includes mortality and CNS disturbance; † losses occurred at third day when house was overheated.

Experiment 3.—The groups were similar to those in experiment 2, except nutrient broth was substituted for the saline diluent and the NDV was from the same lot of virus used in experiment 1 except it had been held at —20 C. for one month. Some preliminary work had indicated that IBV failed to produce marked interference when inoculated with frozen and thawed NDV.

The various groups in this experiment received the following virus exposures: group 1, NDV undiluted; group 2, NDV undiluted and IBV undiluted; group 3, NDV diluted 10^{-5} with nutrient broth; group 4, NDV diluted 10^{-5} with nutrient broth and IBV undiluted; group 5, NDV diluted 10^{-5} in normal a.a. fluid; group 6, NDV diluted 10^{-5} in normal a.a. fluid and IBV undiluted; and group 7, IBV undiluted.

Respiratory symptoms were observed in all groups. Nervous symptoms were evident in all except groups 4 and 7. The daily losses are recorded (table 3). Group 2, which received NDV and IBV undiluted, had a 48 per cent loss compared with a 60 per cent loss in group 1 and failed to show the degree of interference evident in experiments 1 and 2. Dilution of the frozen and thawed NDV with nutrient broth produced a 20 per cent loss in group 4 which indicated interference when compared with no losses in group 3. In group 6, a 32 per cent loss resulted when the NDV was diluted with normal a.a. fluid; this compared with a 24 per cent loss in group 5, which received the diluted NDV alone and indicated no interference. However, there was a delay of a week in the onset of losses in group 6.

At the conclusion of the three-week observation the chicks in group 4, which had received NDV diluted 10^{-5} in nutrient broth plus IBV and had sustained no losses, were inoculated with undiluted frozen and thawed NDV. Group 7, which had received IBV alone, was also inoculated

TABLE 3—Results of Chick Inoculation Studies in Experiment 3

Group	Inoculum	No. of chicks	Symptoms	Losses			Positive HI titers	Positive IB SN titers
				Mortality	CNS dist.*	Total loss** (%)		
1	NDV undiluted	25	R and CNS	13	2	60	5/5	0/5
2	NDV undiluted IBV undiluted	25	R and CNS	11	1	48	5/5	5/5
3	NDV 10^{-5} in (broth)	25	R and CNS	4	1	20	1/5	0/5
4	NDV 10^{-5} in (broth) IBV undiluted	25	R	0	0	0	0/5	5/5
5	NDV 10^{-5} in a.a. fluid	25	R and CNS	6	0	24	4/5	0/5
6	NDV 10^{-5} in a.a. fluid IBV undiluted	25	R and CNS	5	3	32	4/5	5/5
7	IBV undiluted	25	R	1	0	4	0/5	5/5

NDV—Newcastle disease virus; IBV—infectious bronchitis virus; R—respiratory symptoms; CNS—central nervous system disturbances; a.a.—allantoamniotic; HI—hemagglutination inhibition; SN—serum neutralization.

* Chicks with central nervous disturbances evident at end of observation period; ** total loss includes mortality and CNS disturbance.

with NDV to act as a control group. Group 4 had a loss of 70 per cent compared with a loss of 33 per cent for group 7 during a period of 18 days, indicating no immunity to ND resulted from the previous exposure to the two viruses.

Experiment 4.—This experiment was conducted to determine if IBV was capable of interfering when given to chicks prior to exposure to NDV. Twenty-five 4-week-old chicks were exposed to IBV, and one week later they were inoculated with NDV, along with a group of control chicks of the same age. The onset of losses and the daily mortality pattern were approximately the same for both groups. The ND control group had a loss of 76 per cent compared with 68 per cent for the group exposed to IBV and NDV. This would suggest that little or no interference resulted when an interval of one week occurred between the IBV and NDV exposures.

DISCUSSION

Henle,[3] in a review on interference between animal viruses, listed several important factors which exert influence on the reaction occurring in simultaneous exposures. These factors were route of exposure, rate of viral multiplication, and excess of one virus over the other. The viruses in these studies were administered simultaneously in experiments 1, 2, and 3 and by the intranasal route in all cases, eliminating variation by method of exposure. An excess of IBV over NDV resulted in marked interference of NDV. When IBV was in lower concentration than NDV, as in the groups which received the combined undiluted viruses, the IBV was also capable of exerting an interfering influence. The shorter incubation time of IBV compared to that of NDV is probably the most important factor responsible for the interference between these viruses.

The results of experiment 3 indicated that freezing and thawing of the NDV reduced the interference capacity of IBV. However, when the frozen and thawed NDV was diluted (10^{-5}) in nutrient broth, IBV produced complete interference. Dilution of the frozen and thawed NDV with normal a.a. fluid, on the other hand, caused only a temporary interference by IBV and finally a greater loss occurring than in the corresponding NDV control group.

Normal a.a. fluid was used as a diluent to determine whether some factor might be present to influence the reaction between NDV and IBV. Although Hardy and Horsfall [12] stated that allantoic fluid was capable of interfering with the hemagglutination ability of influenza virus, they observed that normal a.a. fluid did not reduce the virulence of the virus for chicken embryos. In experiments 2 and 3, the NDV diluted with normal a.a. fluid caused greater losses in chicks than NDV of equal concentrations diluted with saline and nutrient broth. Dilution of NDV with normal a.a. fluid may have supplied some factor which was more compatible with the multiplication of NDV. In this regard, Beguignon, Guichard, and Vialot [15] found that the addition of normal canine saliva or hyaluronidase to the Louis Pasteur strain of fixed rabies virus resulted in the virus regaining the characteristics of street virus. The group in experiment 2, which received NDV diluted with a.a. fluid and also IBV, showed evidence of marked interference; however, the corresponding group in experiment 3, in which frozen and thawed NDV was used, failed to show evidence of interference.

In experiment 3, group 4, which had previously received NDV (10^{-5}) in broth and also IBV, sustained no losses. However, when this group was inoculated with undiluted NDV three weeks later the loss was 70 per cent. Group 7, the IBV control group, had only a 33 per cent loss when exposed to the same culture of NDV. An explanation for this reaction is suggested in studies of foot and mouth disease made by Verge and Placide.[16] These workers observed that a sensitizing dose of foot-and-mouth-disease virus, administered two weeks before exposure to an infective dose, increased the receptivity of the cattle to foot-and-mouth-disease virus.

SUMMARY

Susceptible chicks were exposed to mixtures of viruses in which the concentration of Newcastle disease virus (NDV) was varied and the infectious bronchitis virus (IBV) concentration remained constant. Results indicated that IBV was capable of interfering with NDV to a marked degree when in excess and to a lesser degree when NDV was in excess.

When NDV had been previously frozen,

IBV was able to interfere only to a limited extent.

Administration of IBV a week preceding the NDV inoculation resulted in no apparent interference.

Newcastle disease virus was more virulent in allantoamniotic fluid than in nutrient broth. Saline as a diluent appeared to have a deleterious effect on NDV.

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Attempts to Influence Listeriosis in Chickens Using Antimetabolites

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THE SUBJECT of this report is an attempt to increase the susceptibility of baby chicks to Listeria infection, by interfering with the metabolism of the host.

Listeriosis of chickens is sporadic and usually affects only a few individuals of a flock. However, heavy losses have been reported in some instances. In several instances other pathogenic agents were also present in the flock.

Paterson[1] reported *Salmonella pullorum* and a heavy tapeworm infection in one flock, with listeriosis and neurolymphomatosis in another. Hurt, Levine, and Graham[2] isolated *Listeria monocytogenes* from 1 bird from a flock where heavy losses had occurred. Coryza had been present in the flock for several months, and ascarids, tapeworms, and coccidia were also found. Bolin and Turn[3] reported isolation of Listeria from a hen from which Newcastle disease virus was also isolated. Lucas and co-workers[4] isolated Listeria from 15 flocks of chickens and in most cases *S. pullorum*, Newcastle disease, helminths, or coccidia were also present. These authors believed that listeriosis in these flocks was usually secondary to some other infection.

Berry and Mitchell[5] found that the administration of different inhibitors and intermediates of the tricarboxylic acid cycle reduced the survival time of mice which had been exposed to *Salmonella typhimurium*. The Salmonella infection which resulted was also more severe as judged by the number of bacteria found in the blood.[6] Gilfillan *et al.*[7] reported similar results in chicks injected simultaneously with *Salmonella pullorum* and certain tricarboxylic acid cycle inhibitors and intermediates. They found that citrate levels in chicks infected with *S. pullorum* were increased slightly during the height of the infection. This indicated a blocking of the enzymes which function in the oxidation of citric acid, with consequent accumulation of citrate. Liver succinoxidase, fumarase, and aconitase were found to be considerably decreased in the experimentally infected chicks. Young and Zelle[8] induced fatal infections in mice, guinea pigs, and rats by respiratory exposure to mixed aerosols of heavy metal salts and anthrax spores in concentrations which would have no lethal effect if used separately. They concluded that the synergistic relationship between the chemicals and the anthrax spores was due to chemical inactivation of enzyme systems dependent upon free sulfhydryl groups for their activity.

MATERIALS AND METHODS

Listeria monocytogenes (strain 1501-2) used in these trials was isolated in this laboratory from the medulla oblongata of a sheep which died with listeriosis in 1951. A suspension of *L. monocytogenes* in sterile skim milk was prepared from 48-hour tryptose agar cultures. Tubes containing 0.1-ml. quantities of the suspension were stored in a mechanical refrigerator at —60 C. The number of viable organisms in the suspension was determined by making plate counts after different intervals of storage. The results of these counts were rather uniform. Each tube contained about 1.2×10^9 viable microorganisms based on the average of 30 counts.

One-week and 2-week-old chickens were used. They were fed a commercial chick starter containing no antibiotics. Exposure was accomplished by intravenous injections of 0.1 ml. of physiological saline containing the appropriate number of *L. monocytogenes* organisms. Sodium salts of succinate, citrate, malonate, arsenite, arsenate, and fluoroacetate and 2,4-dinitrophenol were given to the chickens receiving the different numbers of organisms (table 1). Control chickens receiving *L. monocytogenes* only or chemicals only were included in each experiment.

The succinate, citrate, malonate, and arsenite compounds were administered in approximately the same dosage as used by Berry and Mitchell[5] in mice. Sodium citrate killed chickens when it was injected rapidly but was tolerated when injected very slowly. The experimental injections were given accordingly. The dose of fluoroacetate was one third the $l.d._{50}$ for chickens as indicated by the manufacturer of the chemical.[9] No data on toxicity or dosage of 2,4-dinitrophenol were found in the literature. Enzyme studies[10, 11] have shown that 2,4-dinitrophenol is a powerful inhibitor *in vitro* of aerobic oxidative phosphorylation processes. Green and Beinert[12] found that *in vitro* 2,4-dinitrophenol at M/5000 level (0.037 mg./ml.) almost completely inhibited the phosphorylation process.

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In our experiments, 2,4-dinitrophenol was used at a concentration of 0.1 mg./ml. Although sodium arsenate will interfere with certain steps in glycolysis,[20] no data on toxicity or dosage of arsenate were found in the literature. None of the chemicals had any visible toxic effect on the experimental chickens.

Since in the initial experiment small amounts of chemicals had no effect on the course of listeriosis, larger amounts were used in subsequent experiments. When more than two treatments with a chemical were given, they were administered intramuscularly. In some instances, administration of the chemicals was started 48 hours after exposure in an attempt to first establish the Listeria infection.

Attempts were made to isolate Listeria from liver, heart, and spleen of the experimental chickens. Chickens which lived longer than seven days were killed at different intervals after exposure.

RESULTS

The number of *L. monocytogenes* necessary to produce a fatal infection in 1- and 2-week-old chickens was remarkably uniform. The $l.d._{50}$ for 1-week-old chickens, calculated by the method of Reed and Muench,[14] was 9.3×10^7 microorganisms and for 2-week-old birds it was 4.7×10^7 microorganisms. Sublethal doses failed to produce listeriosis in chickens which received intermediates and inhibitors of the tricarboxylic acid cycle. The survival time after exposure to lethal doses of Listeria was unaffected by administration of the chemicals.

Most of the chicks with fatal infections died less than 48 hours after exposure and none died of listeriosis more than seven days after exposure. Listeria were recovered from the spleen of all, from the liver of all but 1, and from the heart of all but 2 of the chickens which succumbed to listeriosis. *Listeria monocytogenes* was recovered from liver, heart, and spleen of many chickens killed as long as 14 days after exposure and from the spleen of 1 chicken killed 21 days after exposure (table 2). Recovery of microorganisms was most frequent from the spleen, regardless of the dose of Listeria to which the birds were exposed and the time at which they were killed.

DISCUSSION

The main purpose of our investigation was to attempt to increase the susceptibility of young chickens to Listeria infection by interfering with their metabolism. There was no reduction of survival time of the chickens treated with the intermediates or the inhibitors of the Krebs cycle used in this study. Neither did chickens thus treated develop listeriosis following exposure to sublethal doses of *L. monocytogenes*. Treatment with the chemicals used in this investigation failed to influence the course of infection in 1- and 2-week-old chickens.

Different investigators have attempted to produce experimental listeriosis of chickens with various success. Seastone,[15] Graham and co-workers,[16] PeperKamp and Jansen,[17] Pallaske,[18] and Lucas and co-workers [4] were

TABLE 1—The Dosage and Methods of Administration in Treatment of Experimental Chickens with Chemicals Which Are Intermediates or Inhibitors of the Krebs Cycle

No. of birds	Chemical	Dose (mg.)	Administration		
			Method	No. of injections	First injection relative to exposure to Listeria
44	Na succinate	40.0	IV	2 in 24 hr.	Simultaneous
24	Na succinate	80.0	IV	2 in 24 hr.	Simultaneous
28	Na succinate	120.0	IM	6 in 72 hr.	Simultaneous
28	Na succinate	120.0	IM	6 in 72 hr.	48 hr. later
20	Na citrate	20.0	IV	2 in 24 hr.	Simultaneous
12	Na malonate	120.0	IM	6 in 72 hr.	Simultaneous
12	Na malonate	120.0	IM	6 in 72 hr.	48 hr. later
24	Na arsenate	0.2	IV	2 in 24 hr.	Simultaneous
12	Na arsenate	0.3	IM	6 in 72 hr.	Simultaneous
12	Na arsenate	0.3	IM	6 in 72 hr.	48 hr. later
8	Na arsenate	0.6	IM	6 in 72 hr.	Simultaneous
8	Na arsenate	0.6	IM	6 in 72 hr.	48 hr. later
12	Na fluoroacetate	0.145	IM	6 in 72 hr.	Simultaneous
12	Na fluoroacetate	0.145	IM	6 in 72 hr.	48 hr. later
12	2,4-dinitrophenol	3.0	IM	6 in 72 hr.	Simultaneous
12	2,4-dinitrophenol	3.0	IM	6 in 72 hr.	48 hr. later
8	2,4-dinitrophenol	6.0	IM	6 in 72 hr.	Simultaneous
8	2,4-dinitrophenol	6.0	IM	6 in 72 hr.	48 hr. later

IV intravenous; IM intramuscular.

TABLE 2—Isolation of Listeria from Liver, Heart, and Spleen of Young Chickens Examined at Various Intervals After Receiving Different Numbers of Listeria Monocytogenes

No. of bacteria injected	Tissue	Days after exposure				
		2	7	10	14	21
1.2×10^3	Liver	.....	0/4*	.....	.....	0/15
	Heart	.....	0/4	.....	.....	0/15
	Spleen	.....	3/4	.....	.....	0/15
1.2×10^4	Liver	9/10	1/60	0/16	0/70	0/15
	Heart	8/10	7/60	1/16	2/70	0/15
	Spleen	10/10	24/60	5/16	22/70	0/15
1.2×10^5	Liver	.....	2/23	0/14	0/20	.....
	Heart	.....	11/32	0/14	0/20	.....
	Spleen	.....	15/32	2/14	5/20	.....
1.2×10^6	Liver	10/10	15/52	0/16	2/44	0/12
	Heart	10/10	25/52	1/16	4/44	0/12
	Spleen	10/10	29/52	6/16	18/44	1/12
1.2×10^7	Liver	27/28	3/5	0/4	1/13	.....
	Heart	27/28	3/5	1/4	3/13	.....
	Spleen	28/28	4/5	3/4	7/13	.....
1.2×10^8	Liver	39/39	.....	.....	.....	0/1
	Heart	39/39	.....	.....	.....	0/1
	Spleen	39/39	.....	.....	.....	0/1

* 0/4 = no isolations/4 attempts.

able to produce listeriosis in chickens by intravenous inoculation of Listeria, while Paterson [1] stated that Listeria were pathogenic but did not give the method of exposure. Negative results were reported by Schwarte and Biester [19] and Jones [20] with intravenous exposure and by Cole [21] with intraperitoneal exposure. Our experiments indicate that listeriosis can be produced in 1- and 2-week-old chickens by intravenous inoculation of relatively large numbers of the organisms. The $l.d._{50}$ was approximately 1.2×10^5 bacteria and all but 1 of 40 chickens inoculated with 1.2×10^9 bacteria died. The number of organisms used for exposure appears to be a critical factor in the production of listeriosis of chickens. The route of inoculation is also important. Schwarte and Biester [19] produced a fatal disease in 9 of 11 chickens intracerebrally exposed to various doses of Listeria. They were unable to cause listeriosis in 25 chickens by subcutaneous inoculation. In our laboratory, 10 one-week-old chickens were intraperitoneally exposed to 1.2×10^7 and 1.2×10^9 *L. monocytogenes* strain 1501–2 and all survived.

Although in our experiments there is no significant difference between $l.d._{50}$ for 1- and 2-week-old chickens, age is another important factor in experimental listeriosis of chickens. Paterson [22] regularly produced death of chick embryos inoculated with Listeria. In a limited number of trials with strain 1501–2, the $l.d._{50}$ for 7-day-old chicken embryos inoculated into the allantoic sac was 1.8 microorganisms. The $l.d._{50}$ for 14-day-old chicken embryos was 11.0 microorganisms. The extreme susceptibility of the chicken embryo to *L. monocytogenes* may be of value in isolating Listeria from tissues or materials containing small numbers of organisms. On the other hand, older birds seem to be quite resistant. In a number of trials with 2-month-old chickens using 14 different strains, only 2 of 24 birds died following injection of 0.5 to 1.0 ml. of a 24-hour tryptose broth culture of Listeria (approximately 10^9 organisms). Birds surviving the exposure harbored Listeria for a considerable time, particularly in the spleen.

SUMMARY

Several intermediates and inhibitors of the tricarboxylic acid cycle failed to influence the course of experimental listeriosis in young chickens.

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Studies on Errington's Disease in Muskrats
I. Pathological Changes

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ERRINGTON's disease is a highly infectious disease of muskrats (*Ondatra zebethica zebethica*). The first details of its nature and occurrence in the United States were presented by Errington, 1946.[1] His report was concerned with epizootics observed in central Iowa marshes and streams during the years 1943 through 1945. The report included preliminary observations on the gross and microscopic pathological changes of the disease.

Errington's disease is characterized by an acute hemorrhagic inflammation involving one or more portions of the intestinal tract, focal necrosis of the liver, and sudden death of infected animals. Clinical symptoms have never been recorded in animals affected with the disease.

An epizootic occurred among the muskrat population at Horicon Marsh, a state-controlled wildlife area of Wisconsin, in 1946.[2] Findings based on necropsies of specimens in the field led to the conclusion that the Wisconsin epizootic was similar to those observed in Iowa. Detailed studies were not attempted at that time but a project was inaugurated in 1951 to investigate the nature and possible cause of the disease. It is on the basis of these investigations that this report was made possible.

METHODS

Specimens were obtained from animals found dead in the field. The carcasses were shipped to the laboratory immediately, or were stored in a freezer until opportunity for delivery. Gross pathological changes were recorded of all the carcasses, but tissues for microscopic study were taken only from freshly dead, nonfrozen specimens. Tissues were fixed in Baker's formaldehyde. Two staining techniques were employed, hematoxylin-eosin and Mallory's triple stain. The gross and microscopic changes presented in this paper were supplemented by observations made on experimentally produced cases. The descriptions given represent a composite picture obtained from the examination of 66 cases of Errington's disease.

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RESULTS

Gross Pathological Changes.—The gross pathological changes observed in cases of Errington's disease were remarkably uniform. Minor differences were attributed to variations in the severity of the infection and the reaction of the host. External examination of animals which had died of the disease did not reveal significant changes except in those cases in which the colon and rectum were involved. In such cases, the perineal region was blood-stained. Internally, the subcutaneous vessels were injected. The abdominal cavity was found to contain 5 to 15 ml. of blood-tinged ascitic fluid. The gastrointestinal tract revealed the most outstanding changes. The cecum was most often involved and this was followed in frequency by the colon and small intestine. The involved areas were enlarged and acutely hemorrhagic but the serosal surfaces usually were smooth and glistening. Rupture of the gut with resultant peritonitis was observed in one case. Two cases of fibrinous peritonitis were recorded without evidence of previous rupture. The lumen of the involved portion of the intestine contained a chocolate-colored watery exudate. The mucosal surface was eroded down to the tunica propria and in its place was a fibrinonecrotic membrane.

The liver was normal in size, somewhat pale in color, and contained 12 to 50 whitish yellow, circumscribed areas visible on its surface. The cut surface of the liver showed these lesions to be present in the parenchyma of the organ. The size of the lesions varied from 0.5 to 1.5 mm. in diameter. No changes were observed in the gall bladder; the common bile duct was always patent.

The spleen was dark and appeared to be atrophied. The peritoneal surface was never involved and no changes were noticeable on the cut surface.

Pathological changes in the kidneys were not constant and minor alterations in their gross appearance, if present, were invariably masked by autolytic changes. Significant lesions were not encountered in the kidneys in any of the necropsies performed.

The pancreas frequently was enlarged and congested but other alterations were difficult to determine grossly.

Pulmonary changes were those associated with respiratory distress; *viz.*, congestion, with areas of emphysema at the borders of the diaphragmatic lobes. In some specimens, the pathological changes observed simulated those of bronchopneumonia there being a patchy distribution of consolidated hemorrhagic areas. Hydrothorax was a frequent finding.

Pale yellow areas were often observed on the pericardial surface of the heart. Pericarditis was never encountered nor were the valves found to be affected.

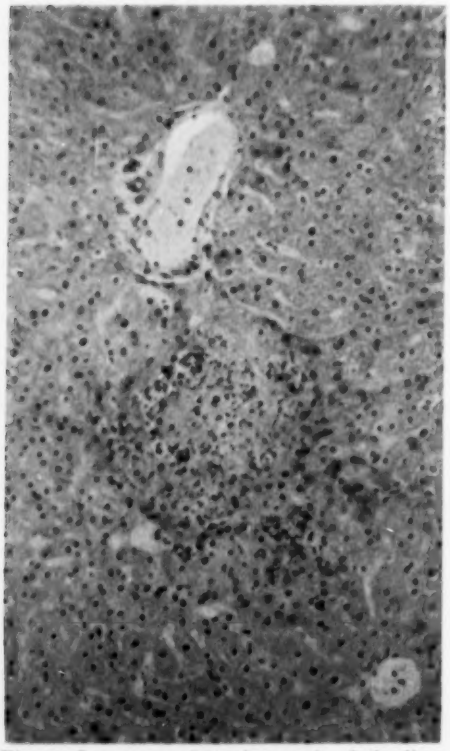

Fig. 1—Low-power photomicrograph of the liver showing focal necrosis. Note the lack of inflammation and widespread pyknosis. x 70.

Benbrook, whose observations were recorded in Errington's report,[1] mentioned the presence of serous peritonitis and pleuritis together with splenic swelling and hyperemia. None of these changes was observed in field or experimental cases. The spleen invariably appeared to have undergone atrophy. Peritonitis, when present, was fibrinous in nature.

Microscopic Pathological Changes.—Tissue sections were prepared from the organs of 19 muskrats which had died from natural or experimental infection.

Liver.—The entire vascular system of the liver was congested. Congestion of the sinusoids usually was severe enough to produce pressure atrophy of the adjacent hepatic cells. This was most noticeable in areas surrounding the central veins. Degenerative changes with minimal mononuclear infiltration were observed around the portal veins and hepatic arteries. None of the bile ducts showed pathological alterations. Throughout the liver, well-circumscribed areas of focal necrosis were observed (fig. 1). These areas presented a characteristic appearance in all the sections studied. They were seen to have an eosinophilic, fibrinoid center containing degenerated leukocytes and hepatic cells. The hepatic cells surrounding the area showed varying degrees of degeneration. Those closest to the center were most severely affected. The cytoplasm of the cells contained large vacuoles and, in other sections, both the cytoplasm and the nucleus showed this change. Toward the periphery of the lesion, the hepatic cells were swollen and frequently contained pyknotic nuclei and hydropic vacuoles. Many hepatic cells adjacent to the necrotic foci contained bacteria in their cytoplasm. In some instances, the bacteria appeared to have bulbous extremities and on two occasions spores were observed (fig. 2). In some tissue sections, well-circumscribed areas were seen consisting of hepatic cells in advanced stages of cloudy swelling with pyknotic nuclei. The cytoplasmic swelling within the cells caused compression of bile canicuili with the result that bile pigment was prominent in all such locations. These areas were presumed to represent the early stages of the necrobiotic process.

An analysis of the hepatic tissue changes

suggested the pathogenesis of the lesions to be as follows. The etiological agent gained access to the liver via the portal circulation. During its passage through the portal triad, it produced degenerative changes in the vessel walls at that point. It then became lodged in the parenchyma where it elaborated toxic substances. These diffused through the tissue to produce degenerative changes with swelling of the cytoplasm and resultant bile stasis. Focal necrosis was the final result.

Spleen.—No changes were observed in the splenic capsule. The organ revealed varying degrees of congestion. In some tissue sections, congestion was perifollicular in distribution. Most of the follicles were no longer visible but the trabeculae were still prominent. Acute inflammatory reactions were not observed. Many macrophages were seen to contain phagocytized blood pigment. The most outstanding change in the spleen was the presence of increased numbers of multinucleated cells throughout the tissue. These cells did not conform to the descriptions given for any of the classical giant cell types and their formation appeared to be the result of incomplete mitosis. This conclusion was based on the finding of incompletely segmented nuclei forming a sausage-shaped band within the cell. The pathological changes observed in the spleen appeared much like those described by Moll,[3] in experimental botulism in mink and ferrets.

Pancreas.—The changes observed in the pancreas were not outstanding, congestion with varying degrees of interlobular and interalveolar edema being characteristic. Many of the alveoli and ducts were filled with pancreatic secretions which appeared to have become hyalinized. In every case there was evidence of degeneration in the islets of Langerhans.

Kidneys.—The microscopic changes observed in the kidneys were not uniform with the exception of congestion which was a constant finding. The renal capsule and epithelium of the pelvis were never involved. A few kidney sections revealed patchy areas of degeneration in the tubules of the cortex and medulla. Calcification was observed in some sections. Fatty degenerative changes in the collecting tubules were present in others.

Intestines.—Regardless of the area of intestine involved, the pathological changes were essentially the same except for varying degrees of intensity. The blood vessels in the muscular layers and submucosa were congested and those of the villi were ruptured with resultant hemorrhage into the lumen of the gut. Hemorrhage was also observed between the muscle fibers of the muscular layers. With the exception of two cases, the serosal surface was not involved. In these 2 animals, a fibrinous exudate was observed with minimal cellular response. Edema of the muscular layers and submucosa was a constant finding. Examination of the mucous membrane revealed degeneration and necrosis but cellular infiltration was not extensive in most of the sections. In severe cases, necrosis extended to the tunica propria leaving cores of villi devoid of epithelium extending into the lumen. A thick necrotic membrane consisting of degenerated epithelial cells, leukocytes, and erythrocytes entangled in a network of fibrin, covered the inner surface of the intestine. In less advanced cases, this membrane was poorly developed. Bacteria similar to those ob-

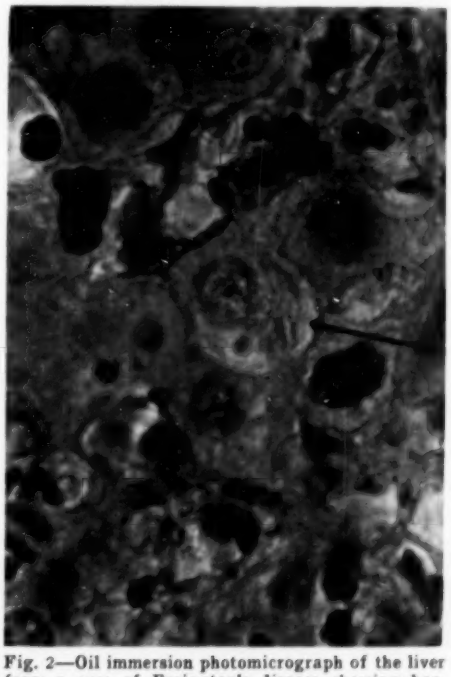

Fig. 2—Oil immersion photomicrograph of the liver from a case of Errington's disease showing bacteria with spores within a hepatic cell. x 1200.

served in the liver were seen in large numbers in the crypts of some of the glands, in the submucosa, and between the muscle fibers. In one specimen, large numbers of the bacteria with terminal spores were observed in the lumen of the submucosal blood vessels. The histopathological changes of the intestinal tract observed among animals which died of Errington's disease were those of a hemorrhagic, membranous enteritis.

Mesenteric Lymph Nodes.—Edema was a frequent finding in the mesenteric lymph nodes examined. This change was most pronounced immediately below the capsule. Large numbers of plasma cells were present. Phagocytosis of blood pigment was frequently observed. Scrutiny of the parenchymal cells revealed varying degrees of degeneration and in one specimen there was a necrotic area histologically similar to those observed in the liver.

The general lack of severe pathological changes of the mesenteric lymph nodes offered support to the proposed hematogenous spread of infection.

Lungs.—Microscopic changes in the lungs were not constant. The most frequent finding was congestion and hemorrhage into the alveoli. Edema, emphysema, and atelectasis were observed in several lung preparations. Pneumonia was not present in any of the sections studied and the bronchi and bronchioles were never involved.

Heart.—Edema and degeneration of the myocardium were constant findings. Myocarditis was never observed.

Brain.—Pathological alterations were not observed in any areas of the brain.

SUMMARY

1) Necropsies were performed on 66 muskrats which had died of Errington's disease.

2) The gross pathological changes were characterized by an acute hemorrhagic reaction in one or more portions of the intestinal tract, focal necrosis of the liver, and atrophy of the spleen. Fibrinous peritonitis was observed in two specimens.

3) The histopathological changes presented in this report were based on the examination of tissues from 19 muskrats which had died from natural or experimental infection. Congestion was common to all of the tissues examined. Well-defined areas of necrosis were present throughout the liver. Hepatic cells adjacent to the necrotic areas contained bacteria with terminal spores. Interlobular and interalveolar edema was present in the pancreas. The islets of Langerhans showed evidence of degeneration. Congestion was the only significant change in the kidneys. The histopathological changes in the intestines were those of an acute membranous enteritis with hemorrhage into the lumen. Degenerative changes were present in the parenchyma of the mesenteric lymph nodes. Subcapsular edema was also observed. There was no evidence of pneumonia in any of the sections studied. A few lung sections revealed edema, emphysema, and atelectasis. Myocardial degeneration and edema were observed in the heart. Histopathological changes were not observed in any of the brain sections studied.

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Studies on Errington's Disease in Muskrats. II. Etiology

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THE GROSS and microscopic pathological changes observed in cases of Errington's disease have been described previously.[1] Acute hemorrhagic, membranous enteritis and focal necrosis of the liver were found to be characteristic lesions in muskrats which had died of the disease. Errington's original report in the *Iowa Quarterly*, 1946,[2] dealt with a description of the morbid anatomy, his attempts to isolate an etiological agent, and a description of the spread of the disease in Central Iowa marshes and streams. The 1946 report indicated that organisms which grew in occasional cultures were later identified as contaminants; *Escherichia coli* was the most common. *Salmonella typhimurium* was isolated from 1 muskrat carcass. Its role, if any, was not determined. The Iowa report did not indicate a specific etiological agent although after failure to isolate a bacterial pathogen the possibility of a filterable virus was considered.

In 1946,[3] an epizootic occurred in muskrat populations in Wisconsin. Findings based on necropsies of specimens in the field led to the conclusion that the Wisconsin epizootic was similar to those observed in Iowa. The Wisconsin report included recorded cases of tularemia and indicated that the fatalities which occurred might have been due to two possible etiological agents, *Pasteurella tularensis* and the agent responsible for Errington's disease. *Pasteurella tularensis* was not isolated from carcasses during the epizootic but two cases in man were recorded in trappers in the vicinity of Horicon Marsh.

The rapid increase in the numbers of muskrats at Horicon, from 1946 to 1951, contributed to the establishment of a project to study the disease; it appeared possible that the new population might again be composed of individuals susceptible to Errington's disease. While an epizootic did not occur, a large number of diseased specimens became available to permit investigations on the etiology of the disease.

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METHODS

Three possibilities, based on the types of lesions observed, the nature of the disease, and the suggestion inherent in the Iowa report, were considered at the beginning of these investigations on etiology. Procedures were adopted leading to recovery of a filterable agent, an aerobic bacterium, and an anaerobic bacterium.

Virus Studies.—Tissue suspensions were prepared from affected livers and affected portions of the intestinal tract of diseased muskrats. The tissues were ground in a mortar with sterile sand; other portions of the same tissues were macerated in a Waring blendor. Two diluents were used: 0.85 per cent saline and a 50.0 per cent mixture of glycerol and saline. The suspensions were filtered through Seitz (L6) filters and the filtrates were held at −30 C. ± until used.

Other liver and intestinal tissue suspensions were treated with penicillin (1,000 units/1.0 ml.) without filtration, the object being to avoid a possible failure of recovery related to the pore size of the filters.

Cecal contents from animals with Errington's disease were suspended in 0.85 per cent saline solution. The suspensions were centrifuged at 3,000 r.p.m. for one hour. Penicillin and streptomycin were added to the supernatant to make final concentrations of 5,000 units and 1.0 mg./1.0 ml., respectively.

Ascitic fluid was recovered from the abdominal cavities of 2 diseased muskrats. The fluid was filtered and used in inoculating embryonating eggs.

A total of 464 embryonating eggs were inoculated with the various preparations described above. The chorioallantoic route was used to inoculate 249 eggs, the allantoic sac for 118, and the yolk sac for 49. Thirty-six eggs were used in screening the suspensions prepared from muskrat cecums; 12 eggs were inoculated with the material filtered from ascitic fluid.

Inoculated eggs were incubated for four days at 37 C. prior to being harvested. The presence of contaminants was tested for at the time of harvest on thioglycolate mediums. Suspected membranes (those showing edema, or small, grayish white opaque areas, or both) were used in serial passages in other embryonating eggs and for inoculation of experimental animals. Portions also

were fixed for the preparation of tissue sections.

Ninety-two white mice, 12 guinea pigs, and 8 hamsters were inoculated by various routes with the same preparations used in inoculating eggs. A number of experimental animals received injections of allantoic fluid and minced chorioallantoic membranes recovered from eggs suspected of having become infected.

A suspension prepared from specimens of *Echinostomum coalitum* present in the intestines of diseased muskrats was injected into 12 mice and 3 hamsters. The trematodes were ground in a mortar with sterile sand and their tissues suspended in 0.85 per cent saline.

Bacterial Studies.—Smears were prepared from washed diseased portions of the intestinal tracts of muskrats which had died of Errington's disease and these were stained with a modification of Gram's stain. Control smears were prepared from animals which had died of other causes.

Tissues from the decomposed carcasses were cultured on six different types of mediums—7 per cent ox blood agar, nutrient agar, Albimi Brucella agar, serum broth, cystine blood agar, and Sabouraud's agar. The inoculated mediums were incubated at 37 C. for seventy-two hours before they were considered to be negative.

Anaerobic cultures were made from those organs showing gross pathological changes. Seven per cent ox blood agar was employed as the medium and incubation was carried out at 37 C. for seventy-two hours in Brewer jars. Affected portions of the intestinal tract were recovered, the mucosal surface washed with sterile saline, and five serial plate dilutions were made. Tissues from nondiseased specimens were cultured in an identical manner. These served as controls.

Muskrat Inoculations.—Forty-nine muskrats were used in an effort to reproduce the disease experimentally. These may be conveniently divided into three groups.

Eight healthy muskrats received preparations each of which was considered to be a likely source of the etiological agent of Errington's disease (table 1). The materials were injected subcutaneously in the interscapular region.

Thirty-two other animals were inoculated with materials recovered from preparations already described.

Four animals were exposed to contaminated cages and 5 to uncontaminated cages in order to establish the importance of body excretions in the transmission of the disease.

TABLE 1—Inoculations of Muskrats with Various Preparations Made from Tissues and Cultures Obtained from Animals with Errington's Disease

Muskrat No.	Inoculums	Results
1	Cecal tissue suspension	Negative
2	Cecal tissue filtrate	Negative
3	Cecal tissue plus penicillin and streptomycin	Negative
4	Liver tissue suspension	Negative
5	Liver tissue filtrate	Negative
6	Liver tissue plus penicillin and streptomycin	Negative
7	Third serial plate aerobic culture (mixed)	Negative
8	Third serial plate anaerobic culture (mixed)	Died of Errington's disease

RESULTS AND DISCUSSION

By none of the methods described was it possible to demonstrate the presence of a filterable agent capable of being cultivated in the developing chicken embryo. As many as ten serial passages were made without establishing an infectious agent. Histopathological examinations of suspected chorioallantoic membranes did not reveal changes common to viral infections. Harvested allantoic fluid was incapable of agglutinating a 1 per cent suspension of muskrat erythrocytes or a suspension of chicken embryo erythrocytes from which the fluid was recovered.

According to Errington,[2] Packer who assisted at the time of the Iowa epizootic was unable to demonstrate a virus by the use of embryonating chicken eggs. Seven- and 8-day-old embryos were used and incubation was continued up to the time of hatching. McLeod [4] stated that the primary cause of the disease was a virus which he was able to isolate and transmit through a succession of animals.

Laboratory Animal Inoculations.—Of all the animals that received injections of the preparations described only 3 mice succumbed. Their livers were found to be spotted with small, white necrotic foci. An epizootic due to infection with *Salmonella typhimurium* was later diagnosed in the mouse colony. It was felt that the lesions observed resulted from infection with this common mouse pathogen.

Errington [2] stated that Packer had attempted to produce the disease in guinea pigs, young albino rats, and mice with negative results.

Bacterial Studies.—Gram-stained smears from diseased portions of the intestinal tract revealed numerous gram-positive rods with terminal spores. Organisms of apparently identical morphology were occasionally found in smears prepared from the liver and spleens. Control smears made from the organs of healthy muskrats and also from those of muskrats which had died from causes other than Errington's disease did not reveal bacteria of the type described.

There was much morphological similarity between the sporeformer observed in smears and those observed in tissue sections of the liver and intestinal tract and described in a previous paper.[1]

Aerobic cultures of the tissues from diseased specimens were negative after incubation at 37 C. for 72 hours on all the different types of mediums employed. The absence of fungous growth on Sabouraud's agar was ascribed to the temperature at which incubation was carried out.

Anaerobic cultures resulted in profuse bacterial growth. It was possible to isolate individual colonies on the fifth serial plate. Cultures obtained from diseased specimens all showed a similar type of growth which differed grossly and microscopically from that obtained from healthy specimens. Gram-stain preparations were made of all the different colonies represented on the plates. The two organisms most commonly recovered were a large gram-positive B-hemolytic rod and a gram-positive rod with a terminal spore. The latter was morphologically similar to the sporeformer observed in smears prepared from the tissues of diseased animals.

Identification of the colonial types was not attempted but pure culture isolations were made of those types that were common to anaerobic cultures from diseased muskrats.

A culture of the sporeformer was sent to James Mandia,[5] Department of Animal Pathology, University of Kentucky, who later reported the organism to be an active saccharolytic Clostridium resembling *Clostridium carnis* but differing in several aspects. It produced acid and gas in glucose, maltose, sucrose, lactose, and salicin. It produced abundant gas in milk without coagulation. It did not liquefy gelatin nor form H_2S and indole. It produced a terminal spore and was nonpathogenic for guinea pigs and white mice.

Muskrat Inoculations.—Only 1 muskrat died of the 8 that received inoculations of materials considered to be likely sources of the etiological agent (table 1). This animal received 1 ml. of the growth obtained from a third serial plate dilution after it had been washed from the plate with 10 ml. of sterile saline. The plate had been prepared from a diseased muskrat, and it had been cultured under anaerobic conditions for 72 hours. It was a mixed anaerobic culture.

A study of table 2 will reveal the numbers of muskrats employed and the types of inoculums administered. Control animals consisted of a representative group of muskrats trapped at the same time, maintained in separate cages but in the same environment, to which nothing was administered. The disease was reproduced on 11 occasions using the following types of inoculums: a suspension containing liver and cecal tissue, fresh fecal filtrate, supernatant fluid recovered from ground chorioallantoic membranes, mixed anaerobic cultures from the cecum, mixed an-

TABLE 2—Summary of Inoculums Used and Results of Attempts to Reproduce Errington's Disease in Muskrats

No. of muskrats	Inoculums used	Route of inoculation	No. animals died / No. inoculated
2	1.0 ml. of suspension containing liver and cecal tissue	IP* and SC*	1/2
1	1.0 ml. fresh fecal filtrate	SC	1/1
1	5.0 ml. ascitic fluid	SC	0/1
2	1.0 ml. chorioallantoic supernatant	IP and SC	1/2
1	3.0 ml. trematode tissue suspension	IP	0/1
3	1.0 ml. cecal tissue suspension	IP and SC	0/3
2	1.0 ml. cecal tissue filtrate	SC	0/2
1	1.0 ml. liver tissue suspension	SC	0/1
1	1.0 ml. liver tissue filtrate	SC	0/1
1	1.0 ml. splenic tissue suspension	IP	0/1
1	0.5 ml. liver—Waring blended—untreated	SC	0/1
1	0.5 ml. liver—Waring blended—filtered	SC	0/1
1	0.5 ml. liver—Waring blended—antibiotic	SC	0/1
1	0.5 ml. cecum—Waring blended—untreated	SC	0/1
1	0.5 ml. cecum—Waring blended—filtered	SC	0/1
1	0.5 ml. cecum—Waring blended—antibiotic	SC	0/1
1	0.5 ml. mixed aerobic culture from cecum	SC	0/1
1	0.5 ml. mixed anaerobic culture from cecum	SC	1/1
2	0.5 ml. mixed anaerobic culture from colon	SC	2/2
2	0.5 ml. hemolytic anaerobic culture from colon	SC	0/2
4	0.5 ml. pure culture of Clostridium	SC	4/4
1	1.0 ml. pure culture of Clostridium	Orally	1/1

* IP, intraperitoneal; SC, subcutaneously.

aerobic cultures from the colon, and pure cultures of the Clostridium.

The length of time that elapsed between exposure and death varied from three to 14 days and generalized edema was more noticeable in experimentally produced cases than in natural infection. The source of the variation in experimental cases may rest in the number of organisms present in the inoculum, in the altered pathogenicity of the organism in the artificial mediums, or in differences in individual animal susceptibility.

TABLE 3—Exposure of Healthy Muskrats to Contaminated and Noncontaminated Cages

No. of muskrats	Condition of cages	No. of deaths due to Errington's disease
4	Errington's disease-contaminated cages	4
1	Errington's disease-contaminated cages treated with 5 per cent creolin	1
5	Noncontaminated cages	0

The death which occurred following the injection of fresh fecal filtrate might be attributed to the presence of a toxin. It is emphasized that fresh material was used in preparing the inoculum. Failure to reproduce the disease consistently by the injection of tissue suspensions and filtrate may have been due to the fact that most bacterial toxins are rapidly destroyed in the body following death. Carcasses were rarely received within 24 hours after death.

In order to establish the role played by body excretions in the transmission of the disease, 4 healthy muskrats were placed in cages that were previously occupied by animals which had died of the disease, and 5 were placed in cages contaminated by muskrats which at the time of necropsy showed no evidence of the disease (table 3). Clean water bowls were placed in both sets of cages. Of the 4 animals, 1 died in six days and the other 3 in seven days with typical lesions of the disease. The species of Clostridium were recovered from the tissues of these animals and also from dry feces taken from two of the cages. No deaths were recorded in the control group.

It was later discovered that the disinfection of cages with 5 per cent creolin was ineffective (table 3). This observation prompted testing a pure culture of the Clostridium in 5 per cent creolin. A 48-hour culture containing large numbers of spores was suspended in 5 per cent creolin for nine days at room temperature. At the end of this time the suspension was centrifuged and washed five times with sterile saline. The sediment was then plated on 7 per cent ox blood-agar and incubated for 72 hours under anaerobic conditions. The organisms were found to be viable even after such treatment. Autoclaving at 250 C. for 20 minutes was found to be the only satisfactory method of sterilizing contaminated cages.

SUMMARY

1) Lack of information concerning the etiological agent of Errington's disease in muskrats prompted investigations that might lead to the solution of the problem.

2) Attempts to isolate a filterable agent by using embryonating chicken eggs were unsuccessful. A total of 464 eggs were inoculated with four different types of inoculums using three routes of inoculation.

3) Albino mice, guinea pigs, and hamsters were not susceptible to a filterable factor.

4) Aerobic cultures from the organs of diseased specimens were negative after 72 hours' incubation at 37 C. Six different types of mediums were employed.

5) A gram-positive, sporeforming bacterium identified as a Clostridium sp. was found to be common to cultures and smears prepared from cases of the disease.

6) The disease was successfully reproduced in healthy muskrats by the use of pure cultures of the Clostridium and also by the use of various inoculums suspected of containing the organism or possibly products of its metabolism. The length of time that elapsed between exposure and death varied from three to fourteen days in experimental animals.

7) Body excretions of affected animals appear to play an important role in the transmission of Errington's disease.

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Studies of Prepared Feeds in Relation to Salmonella Infection in Laboratory Animals. II. Reproduction and Growth of Mice on Heated and Unheated Commercially Prepared Feeds

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FOLLOWING an outbreak in 1949 of an infection due to *Salmonella newport* in mouse-breeding colonies previously free from Salmonella, examination of feed led to the finding of the viable microorganisms in meat scraps and commercially prepared dog and mouse feeds.[1] It seemed useless to attempt to reestablish breeding colonies free from the infection until an uncontaminated feed could be procured. The object of this communication is to report results of comparison of reproduction and growth of offspring in cohort groups from our mouse colony, respectively maintained on different feeds, some dielectrically heated, and some autoclaved. The choice of the feed for the critical part of the experiment was dictated by considerations of commercial availability of a good feed and facilities for dielectric heating in the same place. Our primary concern was not attainment of excellence in nutrition, but rather to eliminate the menace of Salmonella infection in our colony and its interference with experimental work involving the animals. However, it seemed important to consider that inadequacy of a feed might become evident only after several generations had been produced and the offspring raised on it.

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The authors acknowledge the cooperation of the Eastern States Farmers' Exchange in providing the dielectric treatments of feed in their ovens in Buffalo, which was essential to this investigation.

MATERIALS AND METHODS

Heat Treatment of Feeds.—Jungherr[2] in 1950 used dielectric heat in destroying Salmonella and other pathogens in burlap to make used feed bags safe for refilling. The large dielectric ovens described by Flach[3,4] were used in preparing feeds for group 4 in the present experiments. The degree of heating was determined by a series of tests for survival of Salmonella and *Bacterium coli* inoculated into feed cubes and placed in the center of 100-lb. bags of feed. Viable microorganisms having the characteristics of Salmonella were found after heating at 180 F. for 30 minutes but not after like treatment at 215 F. This agrees with Jungherr's findings.[2] Feeds thus heated in bags in dielectric ovens underwent little or no obvious change in physical properties. Thirty minutes between 210 and 220 F. was adopted as standard treatment where dielectric heating was used.

TABLE A—Compositions for the Three Unheated Commercial Feeds

	Feed I (%)	Feed II (%)	Feed III (%)
Crude protein	23.0	25.0	24.0
Crude fat	5.0	8.0	5.5
Crude fiber	6.0	4.0	3.5
Nitrogen-free extract	44.0	....	46.0
	Alfalfa meal	Alfalfa meal	Alfalfa meal
	Corn grits	Cooked corn and wheat flakes	Toasted yellow corn flakes
	Oat middlings	Second clear flour	Rolled oats
	Wheat germ	Bread crumbs	Corn distillers dried solubles
	Dried skim milk	Wheat germ	Dried grain fermentation solubles
	Meat meal	Dried skim milk	Meat meal
	Fish meal	Meat meal	Soybean oil meal
	Liver meal	Fish meal	Dried apple pomace (or citrus meal)
	Soybean oil meal	Liver meal	Fat base
	Steamed bone meal	Soybean oil meal	Brewers yeast
	Dried beet pulp	Ground cheese rind	Vitamin A and D feeding oil
	D-activated plant sterol	Tomato pomace	Irradiated yeast
	Molasses	Rendered animal fat	Vitamin B_{12} and antibiotic feed supplement
	Brewers yeast	Brewers yeast	
	Vitamin A and D feeding oil	Vitamin A and D feeding oil	D-alpha tocopherol acetate
	Vitamin B_{12} feed supplement	Irradiated yeast	Dicalcium phosphate
	Riboflavin supplement	Dicalcium phosphate	Iodized salt
	Thiamin and niacin	Iodized salt	
	Iodized salt	Sodium bicarbonate	
	Manganese sulfate	Chemical preservatives	

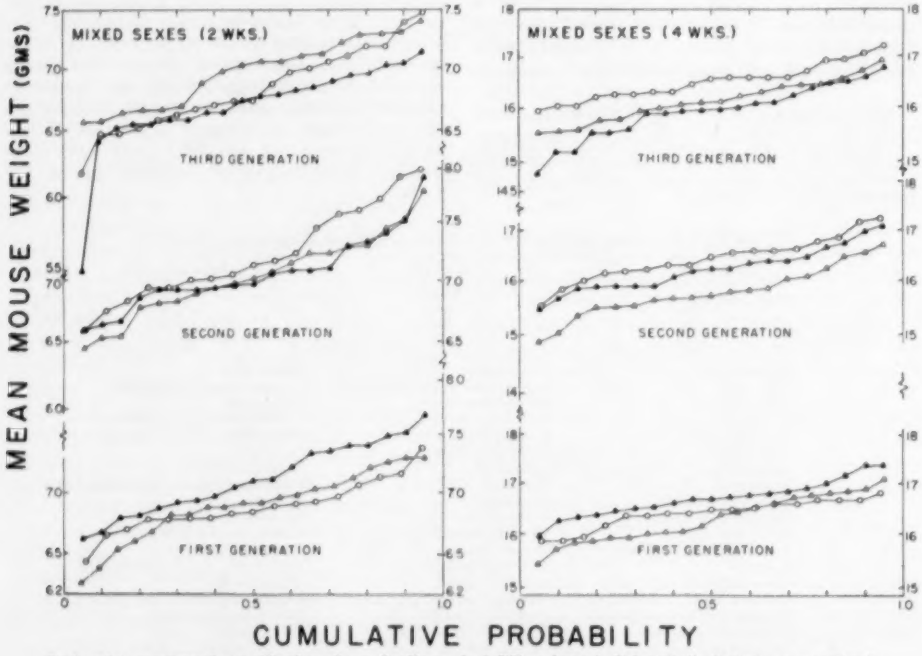
Feed for group 5 was autoclaved at this laboratory, spread to a depth of 2 inches in trays, with a jacket pressure raised to 90 (but not over 95) lb. per square inch and with air exhausted from the chamber by vacuum. Due to inequalities in heat distribution, there was about 10 to 15 degrees difference in temperature of feed in different parts of the chamber; the greatest temperature was attained in the upper trays and a minimum of 100 C. was attained apparently by all the feed. A 30-minute period of treatment was adopted as standard where autoclave heating was used. Some hardening and considerable darkening of feed cubes resulted from this "dry heating" in an autoclave.

Bruce[5] recently reported on the use of an autoclaved stock diet for mice, feed being autoclaved at 15 lb. per square inch for 20 minutes. In a limited number of mice producing five generations of offspring, he found no evidence of impairment of reproduction. However, his observations on growth were restricted to determinations of weight at weaning age.

The compositions of the three feeds used are given in table A.

Maintenance and Observation.—Our primary purpose was to compare reproduction and growth of offspring in three groups, respectively maintained on the same feed (III); either unheated (group 3), dielectrically heated (group 4), or autoclaved (group 5). However, since the feed chosen as readily available in these forms had not been used previously in this laboratory for maintenance of stock mice colonies, we planned to have two other groups simultaneously maintained, respectively, on two other unheated feeds (I and II) that had been so used. Accordingly, five groups of mice from the same generation of the stock colony were formed by distributing litter mates at the time of weaning among the groups. The groups were then subdivided into families, comprised of 1 male and 5 females, which were separately maintained in a 10 by 20-in. box on the same feed as the rest of their group. The plan called for 20 families in each group, but this was not always attained. The families of each group were housed in separate cubicles, but all groups were uniformly treated except for the stipulated difference in feed.

The animals were observed daily, and pregnant females were removed to individual boxes, 6¾ by 7½ in. Litters, alive or dead, the number of young, and the live litter weights at the age of 2, 3, and 4 weeks were recorded. The young were weaned at 3 weeks and the mother returned to her original family box, except that if the young died the breeder was immediately returned.



Each point corresponds to offspring of one family on feed III, unheated, dielectrically heated, or autoclaved as indicated by circles, closed triangles, or open triangles, respectively.

Fig. 1—Mean family mouse weights (mixed sexes) at 2 and 4 weeks of age.

In that case, the unelapsed part of the usual three weeks allowed for weaning was considered as additional breeding time and so applied in the record as an adjustment.

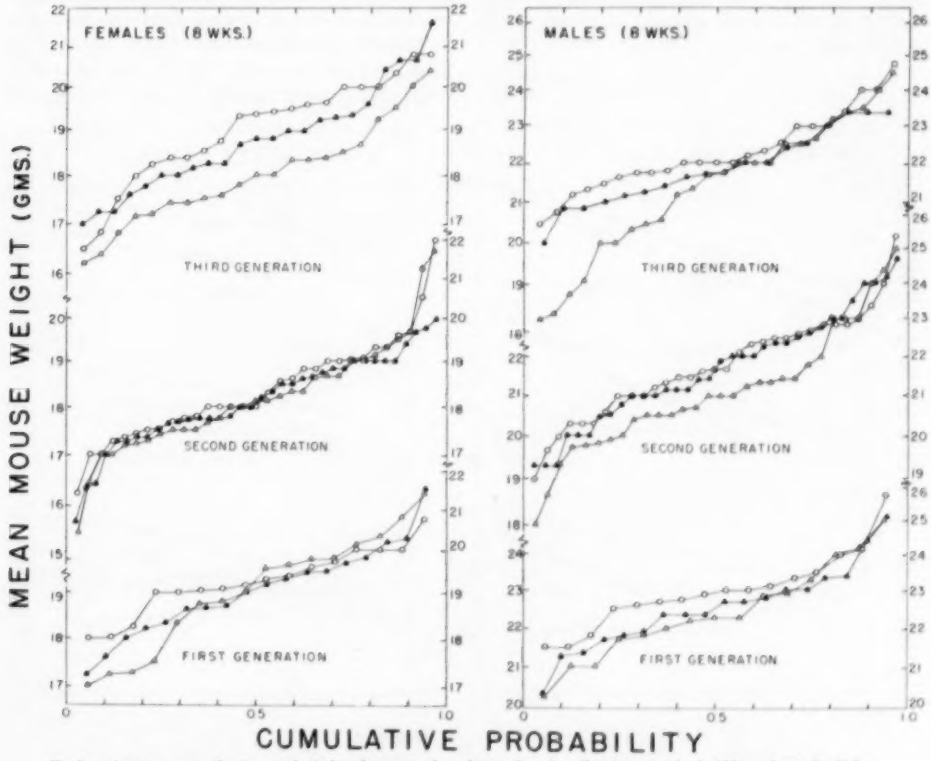
The period taken for observation of breeding of a given family was five months beginning 21 days before its first litter was born. Accordingly, mouse-breeding months were calculated for each family as the sum of breeding months for each of the female breeders (adjusted, if necessary, as noted previously). If there were no irregularities, this would be 25 mouse-breeding months for a family. Offspring born after the actual five-month period prescribed for observation of reproduction were not included.

At the age of 4 weeks a limited number of litters from each group were set aside for further weight recordings. These young mice were assembled in subgroups of 4 to 7 of the same sex and age, and these subgroups were maintained separately on the same feed. The number and total weight of each of the subgroups were recorded at the ages of 4 weeks (when they were assembled) and at 8 weeks. From such 8-week-old mice, random selections were made of breeders for production of the next generation in the experiments. So, in like manner a second and third generation were produced and observed in the respective group categories. A fourth generation was produced under conditions modified to some extent as will be described but, aside from such changes, the original mice of each group and the successive generations were treated as separate colonies, maintained on the feed originally assigned to the group.

RESULTS

Reproduction records of mice on feed III (groups 3, 4, and 5) are shown (table 1). Pregnancies per female mouse-breeding month show a fair agreement for three generations of mice whether on unheated or on heated feed. The number of live young for each group showed variability but not suggestive of detrimental effects from heating feed.

Growth of Offspring.—Growth of young



Each point corresponds to a selected subgroup of male or female offspring on feed III, unheated, dielectrically heated, or autoclaved as indicated by circles, closed triangles, or open triangles, respectively.

Fig. 2—Mean group mouse weights (females and males) at 8 weeks of age.

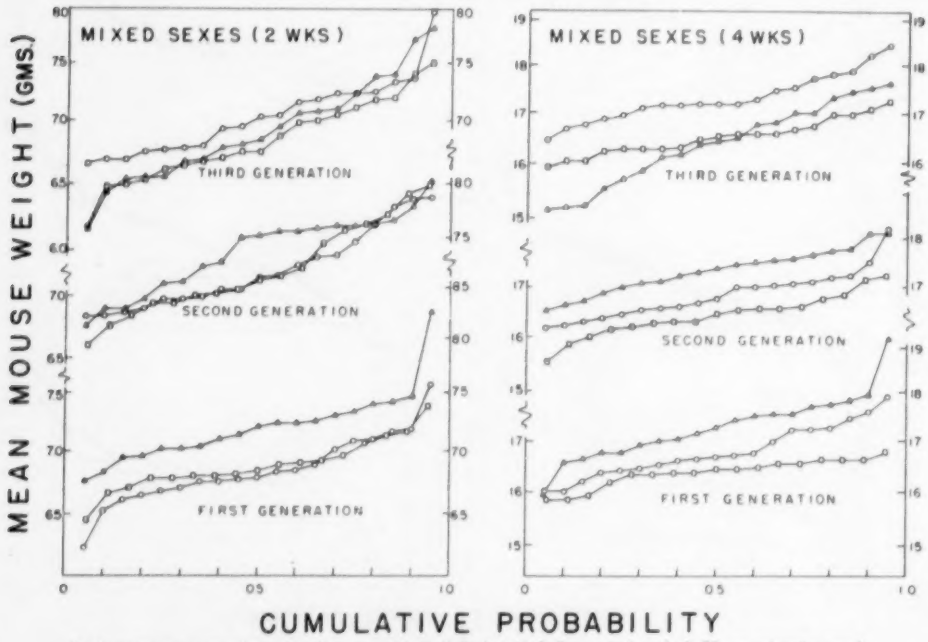
TABLE 1—Reproduction Record of Mice on Feed III, Unheated, Heated in Dielectric Oven, and in Autoclave and Selections for 8-Week Weighings

Families—1 M, 5 F (20 each)	Group-3 mice Unheated feed			Group-4 mice Dielectric heated feed			Group-5 mice Autoclaved feed		
	Gener. 1	Gener. 2	Gener. 3	Gener. 1	Gener. 2	Gener. 3	Gener. 1	Gener. 2	Gener. 3
No. productive	17	17	19	19	19	20	20	19	18
Mouse-breeding months	426.9	426.1	475.1	478.1	479.1	474.5	501.2	473.3	451
Total litters alive 2 weeks	229	209	216	250	243	244	277	230	224
Litters dying or destroyed by mother	30	28	40	38	24	26	27	39	33
Live young per mouse-breeding month	4.15	3.38	3.28	3.61	3.49	3.80	3.76	3.21	3.33
FEMALES AND MALES SELECTED FOR 4- AND 8-WEEK WEIGHINGS									
Females									
No. of subgroups	16	31	21	18	36	23	16	31	21
Total No. of mice	120	194	121	123	208	122	120	204	103
MALES									
No. of subgroups	16	31	23	18	34	18	15	30	24
Total No. of mice	110	157	116	100	171	104	92	123	113

is indicated by mean mouse-weight of family offspring or that of selected male or female groups at given ages. These are shown in graphs on a logarithmic ordinate scale with cumulative frequency as abscissa estimated in accordance with principles developed by Thompson.[6-8] They are briefly as follows:

For n random observations, x_k arranged in order of increasing magnitude, the expectation that a future x under like conditions will be less than x_k is $p_k = k/(n+1)$, which is called the cumulative probability. Here x denotes the mean mouse-weight for a family or a selected subgroup consisting of mice of the same stipulated age from a designated group receiving a specified feed. The graphs are formed in any given case by joining successive points, (p_k, x_k).

The graphs indicate the degree of variability in growth among families or selected



Each point corresponds to families on unheated feeds: feed I as circles, feed II as triangles, and feed III as squares.

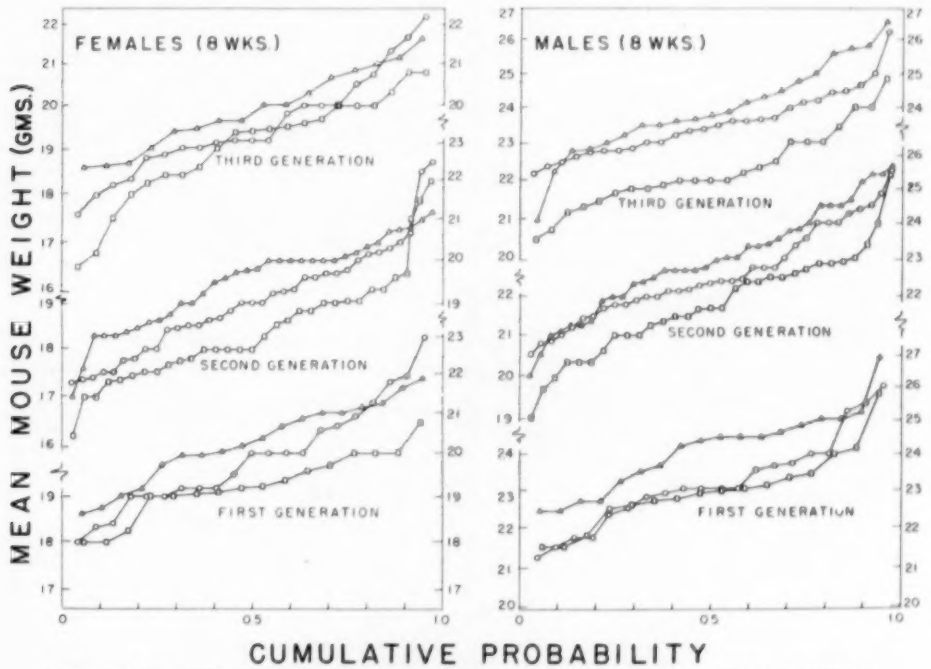
Fig. 3—Mean family mouse weights (mixed sexes) at 2 and 4 weeks of age.

subgroups on the same feed and afford comparison or contrast with other groups under like conditions of maintenance except for use of a different feed. Thus, results are given (fig. 1) for three generations for offspring 2 weeks old on the left-hand chart, and 4 weeks old on the right. Those for unheated feed III (group 3) are shown as open circles, those for dielectrically heated feed III (group 4) are shown as closed triangles, and those for autoclaved feed III (group 4) are shown as open triangles. A like representation is given for corresponding 8-week-old selected subgroups (fig. 2), with females on the left and males on the right.

An appreciation of how small the relative differences are between contrasted graphs may be had by noticing that 0.1 on the abscissal scale (one small scale division) has a length that is equivalent on the vertical scale to about a 4.7 per cent increase, twice the length is equivalent vertically to about a 9.6 per cent increase, and three times the length is equivalent vertically to about a 14.8 per cent increase.

In figures 1 and 2 we may focus attention on the ordinate points on the graphs that correspond to a cumulative probability of 0.5, the mid-points of the graphs. The mid-points of the three compared curves in each case in the two figures, except for 8-week-old females in the third generation, lie within 5 per cent of one another; even in the excepted case, those for unheated and dielectrically heated feed III lie within 5 per cent of each other, and the mid-points for the unheated and autoclaved feed III (open circles and triangles) are less than 8 per cent different. Even throughout the entire curves under comparison most differences do not exceed 5 per cent, rarely 10 per cent. Even in the case treated exceptionally above, the third generation females respectively on unheated and on autoclaved feed III, a statistically significant difference between the graphs (dissociation) is not found in accord with a test described elsewhere.[8]

A corresponding comparison (fig. 3, 4)



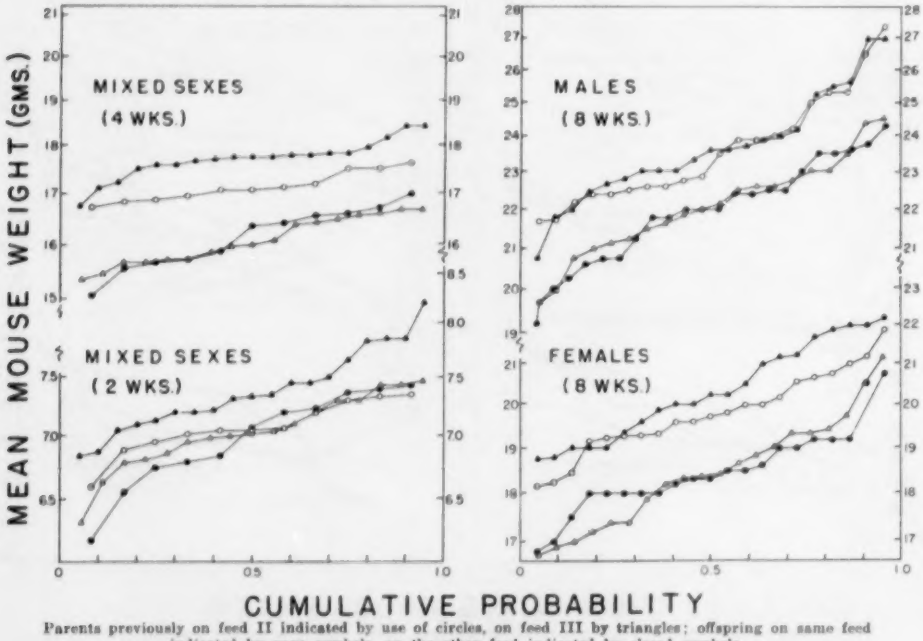
Each point corresponds to selected subgroups on unheated feeds; feed I as circles, feed II as triangles, and feed III as squares.

Fig. 4—Mean group mouse weights (females and males) at 8 weeks of age.

is offered between animals maintained on the three unheated feeds: I as circles, II as triangles, and III as squares—the last being the same as given by circles in figures 1 and 2. The differences in figures 3 and 4 corresponding to use of different feeds are only a little greater than those shown in figures 1 and 2; again 10 per cent or greater differences are fairly rare. However, in ten of the 12 sets of three graphs, the mid-point for feed II is greater than that for either I or III, in one of the remaining two cases the mid-point for II exceeds that for III, in the other case they are nearly the same.

Feed II appears somewhat better than feed III, which had been chosen for the most critical part of the experiment; but to confirm this a fourth-generation experiment was set up. Twenty-four families of third generation offspring on feed II were divided into two groups; group 2a was continued on feed II, whereas group 2b was changed to feed III. Similarly, 40 families of third generation offspring on feed III were divided into group 3a, which continued on feed III, and group 3b, which was changed to feed II. Cumulative probability graphs are shown (fig. 5) for the offspring mean mouse-weights for families at two weeks and at four weeks, and for selected subgroups at eight weeks separately for males and females. Circles are used for animals derived from group 2, open for 2a but closed for 2b; triangles are used for animals derived from group 3, open for 3a but closed for 3b. Thus, the open symbols indicate animals on the same feed as in preceding generations, whereas the closed symbols indicate animals changed to the other feed. These show closer agreement between the animals on the same feed in the current generation than between animals having the same parental group. Thus, the results in figures 3 and 4, indicating somewhat better growth on feed II than on feed III, appear attributable to the difference in feed rather than some inherent characteristics of the respective groups.

Simultaneously with the above, another experiment was set up with other third generation group 2 mice, subdivided into three groups. Of these, group 2 was con-



Parents previously on feed II indicated by use of circles, on feed III by triangles; offspring on same feed indicated by open symbols, on the other feed indicated by closed symbols.

Fig. 5—Mean mouse weights (families at 2 and 4 weeks, groups at 8 weeks) fourth generation.

tinued on unheated feed II, group 6 was changed to dielectrically heated feed II, and group 7 to autoclaved feed II. This is analogous to the experiments with feed III (fig. 1, 2) except that the experiment did not extend into two subsequent generations on the same diet. There was no evidence of better growth on unheated feed II; if anything, the results appear to favor the use of heated feed II.

Discussion

Reproduction records for the other experiments ** were in general harmony with the corresponding growth records as shown in the figures. They indicate a somewhat better reproduction for feed II in all respects than for the other two feeds. For the crisscross experiment with feeds II and III (both unheated), reproduction results were somewhat favorable to feed II. Comparisons of results with the same feed, unheated, dielectrically heated, and autoclaved, respectively, used in simultaneous tests indicate in the case of feed II that there is little or no disadvantage in use of the heated feeds over use of the same feed, unheated, with respect to reproduction; this is in accord with the evidence given in figure 5 with respect to growth. In this case only one generation has been observed, but the results both with respect to reproduction and to growth are in accord with the three-generation experience with feed III similarly compared as to use: unheated, dielectrically heated, and autoclaved as shown in table 1 and figures 1 and 2.

Summary

Heating commercially prepared feeds at 210 to 220 F. in dielectric ovens for a half hour destroyed contaminating microorganisms of the Salmonella group. It did not appreciably impair the nutritive value of such feed for mice, as determined by reproduction and growth of offspring. Tests were carried through three successive generations with feed III and later with only one generation with feed II. The three-generation study was linked to a simultaneous test of two other feeds unheated. The three pelleted feeds were commercially prepared for laboratory mice or for dogs. Feed II, the one that appeared best, was used in the fourth generation to test the influence of preheating. Simultaneously, a crisscross experiment was conducted with some animals changed from feed II to III and some vice versa, with others respectively maintained on their original feed. This showed the better results with feed II to be attributable to the character of the feed rather than to any inherent differences in the groups of animals or from accidental developments in the course of the investigation.

** Tables 2, 3, and 4 have been ozalid reproduced and are available upon request to the authors.

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The Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine

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Literature Cited

In a critical review, Harwood[1] summarized most of the literature on the anthelmintic properties of phenothiazine. He traced its history from a chemical curiosity in 1835 through the discovery of its insecticidal properties in 1934, and finally through its recognition as an anthelmintic in 1938 to the present where it stands as one of the most widely effective drugs available for use in domestic animals.

Phenothiazine is unique in its action in that it acts as an anthelmintic, it suppresses egg production of many nematodes, and also is toxic to free-living stages of nematode larvae in the feces of treated animals.

The first indication that particle size might influence the efficiency of phenothiazine was made by Siegler and Goodhue[2] who reported that two samples containing particles with a mid-size of 4 and 15 μ diameters were more than twice as effective as a sample with a mid-size of 45 μ in diameter. These authors observed the effect of phenothiazine on codling moth larvae when it was ingested on apple plugs. Zukel[3] used preparations containing phenothiazine particles with mean diameters of 3, 10, and 95 μ and showed that the lethal effect was inversely related to the mean diameter when the preparations were applied directly to the cuticle of the cockroach *Periplaneta americana*. He suggested this effect was possibly due to the increased solubility of the smaller particles in the secretions of the dermal glands or in the epicuticle of these insects.

Gordon[4] first reported that particle size affected the efficiency of phenothiazine as an anthelmintic. He found that a finely ground preparation removed 74 per cent of *Oesophagostomum columbianum* from sheep, while a coarse preparation which would pass through a 20-mesh, but not a 30-mesh, screen removed only 38 per cent of the worms. Gordon (unpublished) has extended his observations on particle size to include many kinds of preparations and a number of species of nematode parasites in sheep.

Gibson[5] reported no difference in the efficiency of six samples of phenothiazine of various particle sizes on pure infections of *Trichostrongylus axei* in sheep. This led Jones[6] to conclude that, "The particle size of phenothiazine has no influence on its antiparasitic activity against some and probably against all helminthic parasites." However, the preparations used by Gibson were all what could be termed "fine" since it appears that the preparations had mean particle diameters between 5 and 25 μ. It seems probable that the differences in efficiency between the preparations were so slight that the experimental procedure was incapable of detecting them if they actually existed.

Baker *et al.*[7] reported that in a field trial in which range lambs were drenched with phenothiazine several times over a period of nearly six months, there were no significant differences between the worm burdens of the treated and the untreated lambs. They suggest that the particle size of this material may be an explanation of its apparent failure to bring about a significant reduction of the worm burden. In order to resolve this problem, the experiments reported herein were conducted.

Materials and Methods

The experimental work was undertaken in two phases; in the first, range lambs with natural worm infections were employed and in the second phase, laboratory mice artificially infected with *Nematospiroides dubius* Baylis, 1926, were used as experimental material.[8]

Phenothiazine used was of National Formulary grade, the three formulations being characterized as follows:

1) When wet, the phenothiazine would not pass through a 100-mesh screen, the particles apparently were larger than 140 μ in diameter and is referred to hereafter as "coarse."

2) The commercial N.F. powder, average analysis, showed 20 per cent of the particles larger than 105 μ in diameter, 10 per cent between 44 and 105 μ, 70 per cent smaller than 44 μ diameter. The mean particle diameter of this material was estimated to lie between 40 and 50 μ and is referred to hereafter as "medium."

From the University of California, School of Veterinary Medicine, Davis, and University Field Station, Hopland.

The phenothiazine used in this investigation was supplied by E. I. du Pont de Nemours and Co., Wilmington, Del.

The authors acknowledge their indebtedness to Hugh McL. Gordon of the McMaster Laboratory, Sydney, Australia, who has done much pioneering work on the problem of particle size as it affects the anthelmintic efficiency of phenothiazine. He supplied the authors with his unpublished data which was of inestimable assistance in the conduct of these experiments.

Acknowledgment is also made of the technical assistance of Fay Vance Williams who was largely responsible for the fecal egg counts, the total worm counts, and the identification of collected nematodes.

3) The specially ground commercial N.F. powder had 100 per cent of particles less than 15 μ in diameter. The mean particle diameter was between 1 and 2 μ and is referred to hereafter as "fine."

Fecal egg counts were made employing the Mc-Master technique as described by Gordon and Whitlock[9] and subsequently modified by Whitlock.[10] These counts were expressed in terms of ova per gram of normal feces in the case of the lambs and in ova per gram of dried (to a constant weight) feces in the case of mice.

TABLE 1—Average Fecal Egg Counts of Lambs

Group	Coarse	Medium	Control	Fine
Pretreatment	575	512	569	431
Post-treatment	217	72	453	28
Percentage reduction	62	85	20	93

In the first experiment a group of 40 lambs, averaging 50 lb. in weight, was divided into four equal groups. The grouping was based on repeated fecal worm egg counts in order to assure as far as possible that each group would have approximately the same total worm burden. Over a period of one month while the lambs were kept in a dry corral and fed on alfalfa hay, five pretreatment fecal worm egg counts demonstrated that there were no significant differences between the groups. The lambs were then treated as follows: group 1—25 Gm. of "coarse" phenothiazine; group 2—25 Gm. of "medium" phenothiazine; group 3—no treatment; group 4—25 Gm. of "fine" phenothiazine.

The drug was administered in hard gelatin capsules to insure that each lamb received a full dose.

On the second, fourth, and fifth days following treatment, fecal samples were obtained and worm egg counts made. On the fifth day all lambs were killed. The abomasums and small intestines were removed and total worm collections made. This was accomplished with the abomasum by opening it along its greater curvature. The ingesta was emptied into a pail and the organ was washed thoroughly to free it of worms. The ingesta and washings were passed through a 50-mesh screen, the retained material was washed in water and the residue was transferred to 1-qt. wide-mouth jars to which 5 to 10 cc. of a strong aqueous iodine solution was added.

Worms were removed from the small intestine by first dividing it into approximate thirds and flushing each portion with water under pressure. The ingesta and washings were then handled as described above. Worm counts were made by diluting the total collected material to 1 liter in a cylinder and, after thorough mixing, draining off 100 ml. and counting all of the worms in this portion. This count multiplied by ten gave the total count. The first 20 worms encountered in each sample were removed and subsequently identified.

In the second experiment 40 mice (23 males, 17 females) were each infected with an estimated 100 *N. dubius* larvae and the group was randomly divided into four equal groups. Males and females were maintained separately. Twenty-one days after inoculation, phenothiazine was incorporated into the diets of these groups as follows: group 1—control, normal diet; group 2—0.5 per cent "coarse" phenothiazine; group 3—0.5 per cent "medium" phenothiazine; group 4—0.5 per cent "fine" phenothiazine.

Previous trials had established that 0.5 per cent phenothiazine in the ration of mice did not significantly alter the amount of food ingested. The intake of phenothiazine was estimated to be .04 Gm. per mouse per day.

Pretreatment fecal egg counts were made on the thirteenth and twenty-first day after inoculation of larvae. Egg counts were then made at two- to four-day intervals for 47 days.

Twenty-four days after beginning treatment, the drug was stopped in group 3 (medium) and group 4 (fine). Group 2 (coarse) remained on medicated feed for 45 days. Four mice, 1 from each group, were necropsied on the seventh day of treatment in order to examine the parasites microscopically. Necropsy was performed on all remaining mice on post-treatment day 61 and total worm counts were made.

Experimental Results

Sheep Trials.—Fecal Egg Counts.—The results of five pretreatment and three post-treatment egg counts, given as average number of eggs per lamb (table 1), show that particle size exerts a definite influence on ovogenesis.

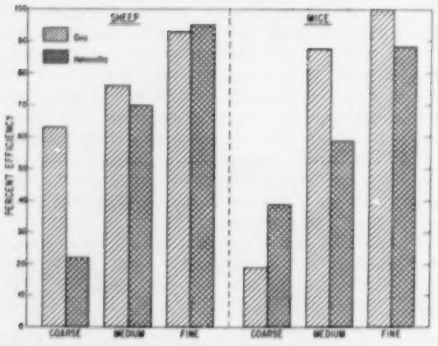
Numbers of Worms in Lambs at Necropsy.—Table 2 shows the average number of worms per lamb recovered at necropsy and table 3 presents these data in terms of percentage reduction of worm numbers with reference to the controls. Analysis of the data indicates that the observed differences are significant at the 1 or 5 per cent level with the exception of the differences between group 1 (coarse) and group 3

TABLE 2—Average Number of Worms Per Lamb

Group	Coarse	Medium	Control	Fine
Abomasum	1,235	460	1,472	53
Small intestine	1,597	677	2,222	144
Total	2,832	1,137	3,694	197

TABLE 3—Percentage Reduction in Number of Worms in Lambs Over Control Group

Group	Coarse	Medium	Fine
Abomasum	16	69	96
Small intestine	28	70	93



Graph 1—The relative efficiency of various particle sizes of phenothiazine in inhibiting ovogenesis and as an anthelmintic.

(control). These differences could be expected to occur by chance between 30 and 50 per cent of the time.

A graphic summary of the data emphasizing the relationship between particle size, ovogenesis, and therapeutic efficiency is presented in graph 1. These data, as they pertain to mice, were arbitrarily selected from the fecal egg counts on the fifteenth day following treatment whereas the data on the lambs represent the average of three egg counts made during the five days following treatment.

Species of Worms Recovered at Necropsy.—Considering only the untreated group of lambs, it is seen from the figures (table 4) that 89 per cent of the worms recovered from the abomasums were Ostertagia. These were largely *Ostertagia circumcincta*, about 2 per cent were identified as *Ostertagia trifurcata* males. Trichostrongylus recovered from the abomasum were approximately 65 per cent

TABLE 4—Percentage of Genera of Worms Recovered from the Abomasums of Lambs

Group	Coarse	Medium	Control	Fine	Ave.
Ostertagia	84	82	89	71	82
Trichostrongylus	16	18	11	29	18

Trichostrongylus vitrinus, 30 per cent *Trichostrongylus axei*, and 5 per cent *Trichostrongylus colubriformis*.

In the small intestines of the untreated lambs (table 5) there were slightly more Nematodirus than Trichostrongylus. The former were approximately 80 per cent *Nematodirus filicollis* and the remainder *Nematodirus spathiger*, while in the latter

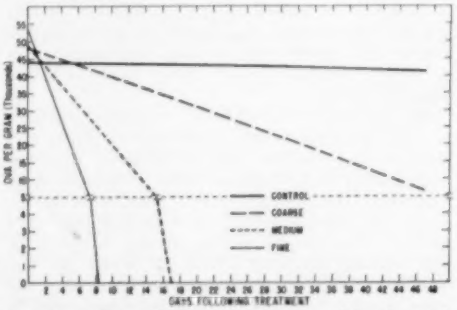
TABLE 5—Percentage of Genera of Worms Recovered from the Small Intestine of Lambs

Group	Coarse	Medium	Control	Fine	Ave.
Trichostrongylus	62	60	48	36	51
Nematodirus	38	40	52	64	49

genus the worms were essentially all *T. vitrinus*.

The well-known variation in efficiency of phenothiazine against various groups of nematodes is shown in tables 4 and 5. It is seen that the ratio of Ostertagia to Trichostrongylus in the abomasums of the untreated lambs is about 8:1 whereas in the group treated with fine phenothiazine it is about 2.5:1. In the small intestines, this differential effect is still present although not so well marked. The ratio of Nematodirus to Trichostrongylus in the untreated lambs is 1:.92 while in group 4 it is 1:.56. From this data it would appear that of the three genera of worms encountered, phenothiazine is most effective against Ostertagia, somewhat less effective against Trichostrongylus, and still less effective against Nematodirus.

Mouse Trials.—Fecal Egg Counts.—The egg counts within a group were found not to differ significantly in males or females. The data, therefore, were combined for analytical purposes. For purposes of comparison, the post-treatment egg counts were converted to linear regression lines by the method of least squares. The standard deviation of these regression lines in thousands of ova per gram is as follows: group 1 (control) 13.7; group 2 (coarse) 13.7; group 3 (medium) 8.0; group 4 (fine) 6.4. This data (graph 2) clearly demonstrates the differential effect of particle size on ovogenesis.



Graph 2—The effect of various particle sizes of phenothiazine on ovogenesis in Nematospiroides dubius.

In order to determine the effect of various particle sizes on the parasite, necropsy was performed on 1 mouse from each group seven days after the experiment began. The effect of the treatment on the egg forming organs is illustrated (fig. 1) showing the posterior portion of a typical female worm from each mouse. It should be pointed out also that the total numbers of worms recovered from these mice were 70, 71, 82, and 72 for groups 1 through 4, respectively. These results demonstrate the difference between the effect of phenothiazine on the worm *per se* and the effect of the drug on the ovogenic structures.

Total Worm Counts.—Necropsy was performed on all mice on the sixty-first day of the experiment. No significant differences were found in the total numbers of worms recovered in male and female mice nor was any differential effect of phenothiazine observed on the male or female worms. The average number of worms recovered in each group is shown in table 6.

The observed differences in worm counts between groups 2 (coarse) and 3 (medium) are significant at the 5 per cent level, the remaining differences are significant at the 1 per cent level. It should be reiterated that the group receiving coarse

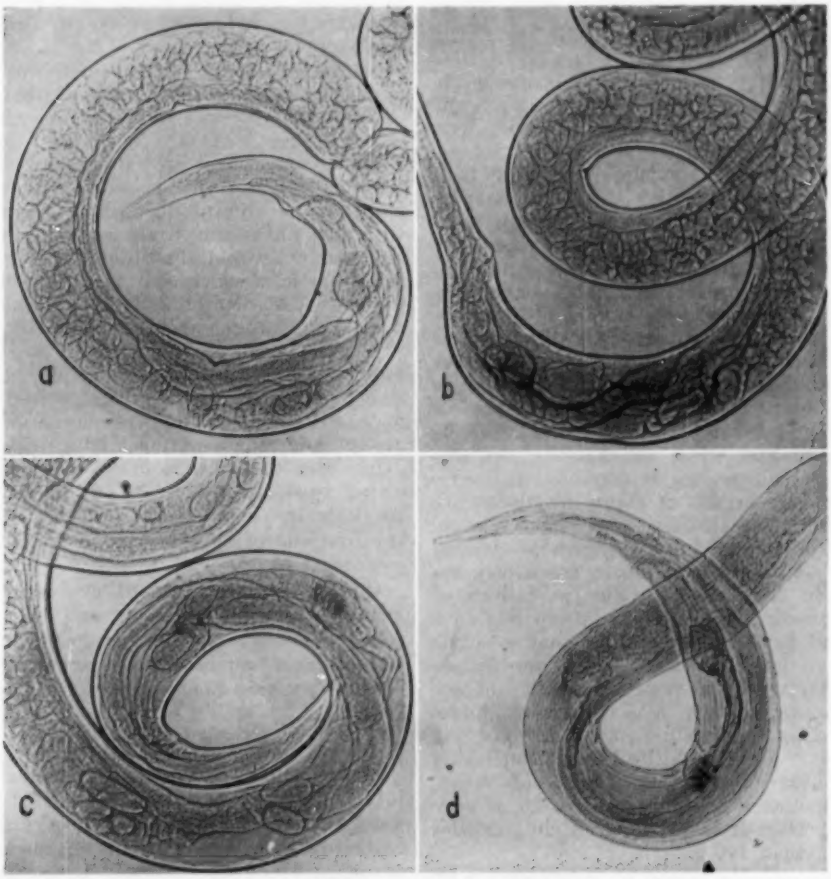


Fig. 1—Photomicrographs of the vulvar region of typical Nematospiroides dubius removed from the mice on the seventh post-treatment day showing the effect of phenothiazine particle size on ovogenesis—(a) no treatment; (b) "coarse" phenothiazine; (c) "medium" phenothiazine; (d) "fine" phenothiazine.

phenothiazine was kept on the drug 21 days longer than groups 3 and 4. It seems likely that had all groups been exposed equally, the difference between groups 2 and 3 would have been even more marked.

DISCUSSION

On the basis of the data presented, it would appear that particle size of phenothiazine exerts a profound influence on the efficiency of the drug; this is most marked

TABLE 6—Average Worm Count of Mice

Group	Treatment	Worm count (ave.)	Percentage efficiency
1	Control	70.7	....
2	Coarse	43.2	39
3	Medium	29.3	59
4	Fine	7.8	89

in the killing effect of phenothiazine on the worm. A less apparent but still significant difference is seen in the effect of the drug on ovogenesis. In the sheep trial, for example, the fine particles were more than four times as effective as the coarse particles in killing nematodes in the abomasum and small intestine; by contrast, the fine particles were only about one and one-half times more effective than coarse particles in inhibiting ovogenesis. These observations are supported by the findings of Lazarus and Rogers[11] who showed that phenothiazine could be found abundantly in the reproductive system of *Ascaridia galli*. While this of itself does not establish a cause and effect relationship, the evidence is suggestive. It may be that the reproductive organs of many nematodes are particularly sensitive to phenothiazine. This is suggested by the common finding that small, repeated doses frequently result in complete reproductive failure of the female worms. In the sheep trials reported here, it is apparent that a single dose of large particles, which were subsequently shown to possess insignificant anthelmintic activity, was capable of inducing a marked reduction in reproductive activity. This would lead to the conclusion that absorption by the parasite of phenothiazine, or a degradation product, is very small when it is administered in particles larger than 140 μ.

Although the action of phenothiazine on the worm is not clearly understood, present evidence suggests that the drug enters the body through the cuticle rather than orally.[11,12] It would seem logical to assume then that since phenothiazine is relatively insoluble in water, and probably also in digestive contents, its action on the worm is by direct solution from the solid state by the cuticle, similar to the phenomenon observed when many insects are exposed to solid insecticide particles.

Collier, Allen, and Swales[13] demonstrated that because phenothiazine is highly insoluble, the rate of absorption by the host is partially dependent upon particle size. The authors showed that in a dose of phenothiazine in which 80 per cent of the particles were less than 4 μ, 10 per cent of the amount administered was excreted unchanged. When a commercial product was administered in which 80 per cent of the particles were over 4 μ, 28 to 36 per cent of the amount administered was excreted unchanged. This suggests that an optimum particle size would be one with a minimal absorption rate by the host and a maximal toxic effect on the parasite. This further suggests the possibility that in addition to eliminating larger particles with low anthelmintic efficiency, a desirable product would also eliminate the very small particles which contribute largely to the rate of absorption and probably not to the over-all anthelmintic efficiency.

A great deal of experimental work is required before the optimum phenothiazine preparation can be characterized. It may well be that an optimum for all groups and stages of nematodes does not exist. For example, it may be desirable, or at least acceptable, to utilize larger particles in prophylactic regimens where suppression of ovogenesis is a major consideration.

On the basis of present evidence, admittedly very scanty, it appears that the most effective therapeutic preparation of phenothiazine would contain particles with diameters within the approximate range of 1 to 25 μ.

SUMMARY

Experiments utilizing natural nematode infections in lambs and artificial infections with *Nematospiroides dubius* in mice demonstrated that the anthelmintic efficiency of phenothiazine is inversely proportional to the diameter of the particles.

Particles larger than 140 μ in diameter were found to be ineffective when adminis-

tered to sheep in a single dose. A preparation with a mean particle diameter between 40 and 50 μ was 70 per cent effective and a preparation with a mean particle diameter between 1 and 2 μ was 95 per cent effective.

Phenothiazine incorporated in the diet of infected mice confirmed the observations on lambs.

It was further shown that suppression of ovogenesis in the nematodes in these experiments was also inversely proportional to the diameter of the phenothiazine particles. In the sheep, particularly, this differential effect was clearly evident.

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The Influence of Environmental Conditions on the Growth and Dissociation of *Brucella Abortus*

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COLONIAL variation in the genus Brucella has been a subject of extreme interest for many years. Investigators have sought to determine the factors that initiate dissociation and to develop methods to prevent its occurrence.

Substances that have been reported to increase the rate of dissociation of Brucella are D-alanine,[6] Mn^{++} and Mg^{++},[5] and fractions I and V of normal bovine plasma.[2] It has been noted that dissociation of Brucella is suppressed by normal serums from Brucella-susceptible species,[3] by γ- and certain β-globulin fractions of normal bovine plasma,[2] and by sodium pyrophosphate.[8] According to Braun,[1] environmental conditions which decrease the growth rate of *Brucella abortus* lower the dissociation percentages and the growth rate and viability of a variant determine its chances of establishing itself within a population after the population pressure has started.

In this laboratory it has been observed that dissociation of Brucella cells when grown in a tryptose medium containing glucose in stationary tubes is rapid and multiplication of cells is slow. In other environmental conditions, dissociation of Brucella cells is extremely slow in rapidly growing cultures which reach high total cell counts.[10]

This study was initiated to determine by reproducible methods of measurements the effect of glucose and other environmental factors on the occurrence of variation in smooth and nonsmooth colonial types of *Br. abortus*.

MATERIALS AND METHODS

Cultures.—Three aerobic strains of *Br. abortus* were studied: smooth type, 2308; smooth-intermediate$_1$ type, 3010; and smooth-intermediate$_2$, 1336. The strains were maintained as pure colonial types on tryptose agar plates as described by Huddleson.[9]

Single colonies from the tryptose agar plate were streaked on liver agar slants and incubated at 37 C. for 48 hours. The growth on the slants was suspended in diluting fluid (0.05% tryptose peptone and 0.5% NaCl) and the density adjusted to a standard turbidity. Ten milliliters of the liquid medium were inoculated with 0.2 ml. of the cell suspension containing 7.5×10^7 viable cells per milliliter.

In discussions, smooth (S) includes the smooth-intermediate (SI) types of colonies and nonsmooth (non-S) refers to all rough, mucoid (M), and sector types of colonies.

Medium.—The base medium contained 2 per cent tryptose peptone, 0.5 per cent NaCl, and 0.5 mg. of thiamin hydrochloride per 100 ml. It was adjusted with H_2PO_4 to obtain a pH of 6.8 after autoclaving.

Various substances were added to the base medium. Unless specified, the addition of these substances was made before autoclaving.

The mediums were distributed in 10-ml. amounts in test tubes (15 x 1.5 cm.) and in Erlenmeyer flasks (50 ml.) and autoclaved at 115 C. for 20 minutes.

Procedures.—The mediums, after inoculation with cells of a pure colonial type of *Br. abortus*, were incubated at 37 C. and examined in duplicate at two-day intervals for eight to 12 days or at the end of seven days.

The total cell count per milliliter of each culture was determined turbidimetrically from a standard curve and pH was measured electrometrically.

Tryptose agar plates were flooded with 0.5 or 1.0 ml. of each of four to seven dilutions of the cultures and incubated at 37 C. for four days. The viable cell count and the extent of dissociation were determined from the colonial growth. Only those plates showing 100 to 600 evenly distributed colonies were examined. The total number of colonies on each plate was recorded and the number of non-S colonies was determined after staining the colonies with an aqueous solution of crystal violet (1:2000).[11] The dye solution was decanted and the colonies immediately examined under a low-power microscope by obliquely reflected light. Nonsmooth colonies were readily differentiated from S and SI colonies by the characteristic colors produced by the staining. The characteristic colors of colonial variants were established from studies of pure colonial types identified on tryptose agar plates according to Huddleson[9] by shape, diameter, opacity, color, consistency, acriflavine spot tests and agglutinability by specific serums. Smooth and SI colonies, stained with crystal violet, vary from a greenish yellow to a reddish yellow and can not be distinguished unless the

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colonies are adjacent. One group of related mucoid types is seen in various shades of pink in the center with blue to purple border and a second group in shades of reddish purple. The sector smooth-mucoid colonies show alternating triangular sectors of yellow and purple. Rough types of colonies are shades of blue-violet; the surface of some shows cracks. The several types within each related group of mucoid and rough colonies may be distinguished only when the agar plate is seeded with a cell suspension sufficient to produce 100 to 200 colonies of maximum size; variations in hues of colors are not easily detected in small colonies.

TABLE 1—The Effect of Agitation and Aeration of Liquid Medium on Growth and Dissociation of Brucella Abortus S Type

Conditions of incubation [a]	Non-S (%) [d]	Total cells (10^9/ml.)
Stationary	74.7	3.9
Shaken [b]	42.6	5.6
Aerated [c]	0.1	41.7

[a] 37 C. for 7 days in test tubes of tryptose-glucose medium; [b] shaken once each day by hand; [c] oxygen passed continuously through medium; [d] viable cells.

The entire plate was examined after staining with crystal violet and all S or all non-S colonies were counted. The different colonial variations represented in each culture were noted but attempts were not made to record the colonial type of each non-S colony.

In this study it was not possible to distinguish certain mucoid types of colonies from S or SI types when they were widely separated without the employment of the crystal violet staining technique.

RESULTS

Effects of Aeration and Agitation.—The tryptose-glucose-thiamin liquid medium is satisfactory for the rapid growth and high cell populations of pure colonial types of Brucella in large quantities (1 to 6 liters) only when the medium is agitated or aerated with a stream of air or oxygen. Attempts were made to create similar environmental conditions in test tubes.

Tubes of inoculated tryptose liquid medium were aerated by shaking once each day during the period of incubation (table 1). After seven days, the percentage of non-S colonial types arising from the S-type strain was 42.6 as compared to 74.7 in an inoculated stationary tube of the same medium. More effective aeration of the inoculated medium in test tubes was achieved by passing oxygen into the medium slowly through a 3-mm. glass tubing with a capillary tip at the bottom of the test tube. To decrease foaming, aerosol O.T. was added to the medium (1:500,000). In this concentration in stationary cultures, aerosol O.T. exhibited no effect on the growth or development of variants from strain 2308 (S). After aeration with oxygen for seven days, less than 0.1 per cent of the viable cells were non-S variants and the total cell count was 42×10^9 cells per milliliter.

Effect of Depth of Medium.—The growth and dissociation of S and SI-type strains were observed in tryptose-glucose medium in several types of containers chosen to provide various depths of the liquid medium and various surface areas. The number of cells introduced into each container of medium was proportional to the volume of medium.

In each container of a shallow layer of medium (0.8 cm.), cell multiplication of the S type was rapid and the differential colonial counts showed less than 10 per cent non-S types after incubation for seven days (table 2). In a deep-layer medium (3 to 8 cm.) the cells grew slowly and more than 60 per cent of the colonies that developed from platings of the medium were non-S types.

The growth rates of the SI strains and the appearance of non-S types in the medium in various containers were not significantly different from those of the S strain; an increase in the depth of the medium resulted in less growth and more rapid dissociation to non-S types.

The data in table 2 include comparisons of growth and dissociation in medium of the same depth but of different surface areas (50- and 500-ml. Erlenmeyer flasks) in addition to medium of different depths but of the same surface area (125-ml. pyrex dilution bottles). A shallow layer, not a large surface area, was shown to be the important factor in obtaining fast mul-

TABLE 2—The Effect of Depth of Liquid Medium in Various Containers on Growth and Dissociation of Brucella Abortus S Type

Container	Depth of medium (cm.)	Non-S (%)	Total cells (10^9/ml.)
Test tube	5.5	74.7	3.9
Dilution bottle	6.0	88.3	1.9
Dilution bottle	3.2	75.8	6.0
Dilution bottle	0.8	7.7	16.9
Erlenmeyer, 500 ml.	0.8	5.6	20.8
Erlenmeyer, 50 ml.	0.8	0.4	32.4

Incubation at 37 C. for 7 days in tryptose-glucose medium.

tiplication and slow dissociation of cells to non-S types in stationary containers.

The rapid multiplication of the cells and the low rate of dissociation in liquid medium in 50-ml. Erlenmeyer flasks (depth 0.8 cm.) paralleled the findings obtained with cells grown in the aerated (oxygen) cultures.

Effects of Various Agents.—Experiments to determine the effect of glucose, fructose, sucrose, and DL-alanine in the tryptose liquid medium on dissociation were conducted in test tubes and 50-ml. Erlenmeyer flasks. The carbohydrates were autoclaved in 20 per cent solutions and added to sterile tryptose medium.

The results in table 3 show that the percentage of dissociation of S-type strain after incubation for seven days varied considerably in the presence of the agents but, in each, the cultures in medium of a shallow layer in the flasks exhibited considerably less dissociation than those in the same medium of a deep layer in test tubes. When test tube cultures containing DL-alanine were aerated (oxygen) during incubation, little or no dissociation to non-S types occurred. In stationary cultures grown in the presence of glucose and DL-alanine in test tubes, the viable cells were 97 per cent non-S types as compared to 0 per cent in the same medium which was aerated (oxygen) in test tubes. The total cell counts of the cultures in stationary flasks or aerated in test tubes were always greater than those in stationary test tubes. The degree of dissociation that occurred in cultures in stationary test tubes to which DL-alanine was added was far less than that which occurred in cultures in medium containing glucose and DL-alanine.

Effects of Serum.—Braun [3] studied the effect of serums from different species of animals upon the variation of *Br. abortus* and found that serum from normal cows completely suppressed dissociation of strain 19 and a virulent S type in broth cultures. He reported that serum from Brucella-infected cows failed to suppress dissociation of the virulent S-type *Br. abortus* but did suppress the dissociation of strain 19.

The effect of an adequate supply of oxygen on the appearance of non-S variants from S-type cells in the presence of different serums was studied briefly. Serum was obtained from the blood of a normal rabbit and from a cow naturally infected with Brucella. The agglutination titer of the bovine serum was 1:10,000. The serums were filtered through a Hormann D-8 pad and added to the sterilized mediums to a final concentration of 5 per cent. After incubation of the normal serum-broth cultures for seven days, no variants were recovered from the cultures in flasks and less than 1 per cent were recovered in cultures in test tubes in medium with or without glucose. Growth rate of the S cells was not altered by normal rabbit serum in either medium in tubes or flasks. The results observed when 5 per cent anti-Brucella bovine serum was added to the mediums was not significantly different from those containing normal rabbit serum: dissociation of S cells was suppressed by the anti-Brucella serum. In tubes containing tryptose-glucose medium, only 3.7 per cent variants were observed in the presence of the anti-serum as compared to 75 per cent in the control culture with no serum. Rapid growth of S type in glucose medium in a shallow layer in flasks was

TABLE 3—The Effect of Various Agents in Liquid Medium on Growth and Dissociation of Brucella Abortus S Type

Agent	Container: Test tube [a] Non-S (%)	Test tube [a] Total cells (10^9/ml.)	Test tube [a] Viability (%)	Flask [b] Non-S (%)	Flask [b] Total cells (10^9/ml.)	Flask [b] Viability (%)
Tryptose base	3.8	1.6	44	0.5	7.9	57
Glucose, 1.0%	74.7	3.9	41	4.3	32.4	53
Fructose, 1.0%	78.6	3.0	43	10.2	21.4	69
Sucrose, 1.0%	17.0	1.6	44	0.8	8.4	49
DL-alanine, 0.2%	25.7	1.1	28	0.7	6.9	48
DL-alanine, 0.2%; aerated [c]	3.9	5.8	9	....	....	....
DL-alanine, 0.2%; glucose, 1.0%	97.4	5.2	38	7.4	27.5	10
DL-alanine, 0.2%; glucose, 1.0%; aerated [c]	0.0	38.5	17	....	....	....

Incubation at 37 C. for 7 days in tryptose medium plus agent; [a] depth of medium 5.5 cm.; [b] depth of medium 0.8 cm.; [c] oxygen passed continuously through medium.

inhibited by anti-Brucella serum: the total cell counts were approximately one-half that of the control.

Growth of Nonsmooth Types.—Five different non-S colonial types derived from the S strain were studied to determine whether or not certain environmental conditions inhibited rapid multiplication of non-S cells. Each of the pure non-S types was inoculated into separate test tubes and Erlenmeyer flasks containing the base tryptose medium and into separate ones containing the same medium to which was added 1 per cent glucose. Examinations were made at two-day intervals as previously described. The findings (table 4) show that the same general growth response was obtained with the five non-S colonial types as with the S type. Each non-S type grew more rapidly in flasks than in tubes and grew more rapidly in medium containing glucose than in one without glucose. However, in the glucose medium in test tubes, the total cell counts of each of the five non-S types was higher than cultures of the S type. The reverse was found in flasks; the growth of the S type surpassed that of the non-S types.

Other variants arose from the non-S cultures in the presence of glucose in the medium. The number of new variants was greater in the Erlenmeyer flasks than in test tubes.

Growth of Mixtures of Smooth and Nonsmooth Types.—In order to determine if the apparent differences in growth of the S and non-S types in different environmental conditions were influenced by the composition of the initial inoculum, medium was inoculated with known mixtures of cells of a smooth and a mucoid type and examined at two-day intervals. In the tubes of tryptose-glucose liquid medium inoculated with 100 per cent S, with 90 per cent S and 10 per cent mucoid, or with 15 per cent S and 85 per cent mucoid cells, after incubation without agitation for 12 days, there were 5.0–5.9 x 10^9 cells per milliliter of which 92 to 100 per cent of the viable cells were non-S types (fig. 1). In Erlenmeyer flasks, however, the liquid medium inoculated with the same proportions of cells as mentioned for the test tubes, and incubated in the same manner, contained 20.6–26.6 x 10^9 cells per milliliter of which only 4 to 16 per cent of the viable cells were non-S (fig. 2). No S or SI types were found in the medium inoculated with 100 per cent mucoid type cells.

The presence of 10 per cent mucoid cells in the inoculum decreased the initial multiplication rate of S cells in the test tubes but had no effect on the initial or maximum growth of S cells in flasks.

TABLE 4—Comparison of Growth and Dissociation of Brucella Abortus S Type with Five Pure Non-S Types

Colonial type	Container	Total cells (10^9/ml.)	Variants (%)
S	Test tube	4.0	84
	Erlenmeyer	38.2	0
Non-S_1	Test tube	14.0	67
	Erlenmeyer	31.9	90
Non-S_2	Test tube	5.9	0
	Erlenmeyer	33.6	5
Non-S_3	Test tube	5.0	1
	Erlenmeyer	23.8	3
Non-S_4	Test tube	15.3	1
	Erlenmeyer	31.4	44
Non-S_5	Test tube	12.3	0
	Erlenmeyer	31.9	48

Incubation at 37 C. for 8 days in tryptose-glucose liquid medium.

The presence of 15 per cent S cells in the inoculum decreased the initial multiplication rate and total production of mucoid cells in flasks, but had no effect on the initial growth or maximum number of mucoid cells in test tubes.

Viable cell counts of cultures in stationary test tubes show that the maximum number of viable S-type cells (0.2–0.4 x 10^9/ml.) occurred after two to four days of incubation. These maximums were noted at the same time intervals in cultures inoculated with 100 per cent S cells, with 90 per cent S and 10 per cent M, or with 15 per cent S and 85 per cent M. In each of these cultures, and in addition to those inoculated with 100 per cent M cells, the maximum number of viable non-S cells (1.7–2.0 x 10^9/ml.) occurred after incubation for nine days. In the culture inoculated with 100 per cent S cells, the decrease in the number of viable S cells was noted before or at the time that non-S cells were detected. In all cultures in flasks, maximum viable cell counts of S and non-S types were noted after incubation for six to eight days.

DISCUSSION

In the base tryptose liquid medium without glucose, in test tubes, the multiplication rates of S and SI types of *Br. abortus* were slow and unless the cultures were in-

cubated for a long time, dissociated types evolved only in small numbers. Several agents cause an increase in non-S cells from S or SI-type cells grown in liquid medium in test tubes, or when grown in liquid medium of a deep layer in other containers. The presence of either of two agents, glucose and fructose, caused an increase in the total cell production. Other agents, sucrose and DL-alanine, in the concentrations used, failed to influence total cell production during seven days of incubation.

Goodlow *et al.*[6,7] demonstrated the toxicity of D-alanine for S cells and the accumulation of this metabolite in liquid medium cultures of Brucella. The authors stated that the accumulation of alanine limits the increase of viable alanine-sensitive S cells and creates an environment favorable for the progressive establishment of alanine-resistant R cells. According to Braun *et al.*,[4] the initial proportion of a given mutant in a mixed population will determine the ability of the mutant to establish itself because of quantitative differences in alanine production and selective value of alanine-sensitive cells.

The dissociation of S and SI types of *Br. abortus* can be prevented or greatly reduced in the presence of any of the previously mentioned agents by increasing the supply of air or oxygen to the growing cultures. This was accomplished by agitation, aeration with pure oxygen, or by growing the cultures in a shallow layer of liquid medium. It was shown that in tryptose-glucose liquid medium in test tubes (depth of medium 5.5 cm.) after incubation for 12 days, the cell population was 97 to 100 per cent non-S types, irrespective of the ratio of S to non-S in the inoculum; in comparable cultures in Erlenmeyer flasks (depth of medium 0.8 cm.), the cell population was only 4 to 16 per cent non-S types.

The growth conditions which favor stable S and SI colonial types are also suitable for rapid multiplication. Total cells in the presence of fructose and glucose numbered 20–30 x 10^9 per milliliter while the total number of cells in the presence of sucrose or DL-alanine was not different from that obtained in the control cultures in a shallow layer. At the same time, neither of these agents caused an increase in dissociated types in cultures in a shallow layer: less than 1 per cent of non-S types of cells were found after seven days. DL-alanine in the liquid medium does not alter the enhancement of growth of *Br. abortus* by glucose. In the presence of these two agents, dissociation of S cells was rapid in medium in test tubes: after seven days, 97 per cent of the viable cells were non-S types. In the medium in Erlen-

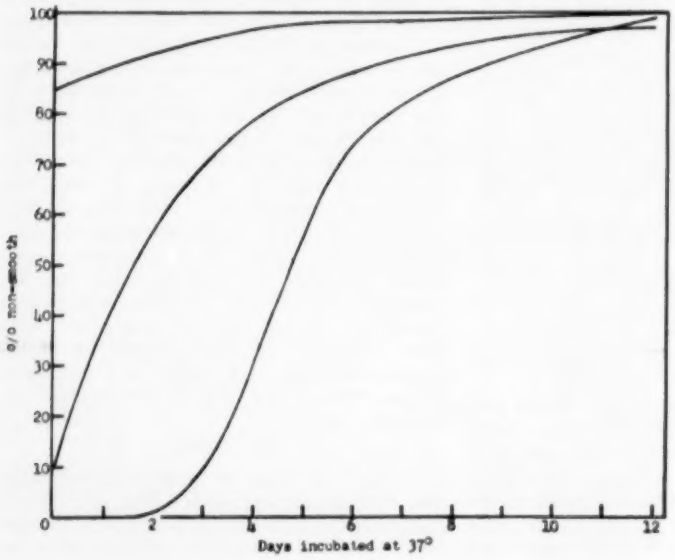

Fig. 1—The percentage of nonsmooth colonial types of Brucella abortus in tryptose-glucose liquid medium in test tubes.

meyer flasks, however, there were only 7 per cent non-S types.

Braun [1] found that the "dissociation index" of *Br. abortus* strain 19 in a daily disturbed population was much lower than the dissociation index of the genetically identical population left undisturbed for ten days. He reported that the total number of cells after daily disturbance was smaller than the number in equally old but previously undisturbed cultures. The differences in growth were accounted for by temperature changes to which the disturbed cultures were subjected when they were removed daily from the incubator for streaking, and the lower dissociation index was a result of less growth.

Goodlow *et al.*[6] mentioned briefly that different results were obtained when cultures were maintained in tubes, flasks, bottles or when static or aerated cultures were used. It was stated that the reasons for such differences were somewhat obscure but could be associated with the ratio of quantity of mediums to the surface area, which controls the available oxygen.

It has been shown in this study that adequate oxygen in a liquid medium permits S cells to multiply rapidly and with little or no dissociation to non-S cells in the presence of substances utilized (glucose and fructose), in the presence of substances not utilized (DL-alanine and sucrose), or in the base tryptose peptone medium. Adequate oxygen facilitated more complete utilization of glucose and fructose and altered the accumulation of metabolic products in the medium.[10] There is no evidence, however, that oxygen initiated utilization of DL-alanine and sucrose; a comparable increase in multiplication rate of S cells occurred in medium in shallow layer in flasks when neither of these agents was added to tryptose peptone medium. Therefore, an adequate supply of oxygen is essential for the rapid and continued multiplication of S cells and, when supplied, eliminates the effects of toxic substances in a culture.

It has been shown that a high oxygen concentration in liquid medium does not create unfavorable conditions for rapid growth of non-S types of cells. Pure cultures of non-S types of cells grew rapidly in shallow layers of tryptose-glucose liquid medium; total cell production in a shallow layer of medium was from 2 to 6 times greater than in a deep layer of medium. When S-type cells and non-S cells are growing together in liquid medium in a shallow layer in stationary cultures, there is a marked depression in the multiplication rate of non-S cells. This finding parallels the depression of growth of S cells in cultures in test tubes in the presence of non-S types. The agent or agents responsi-

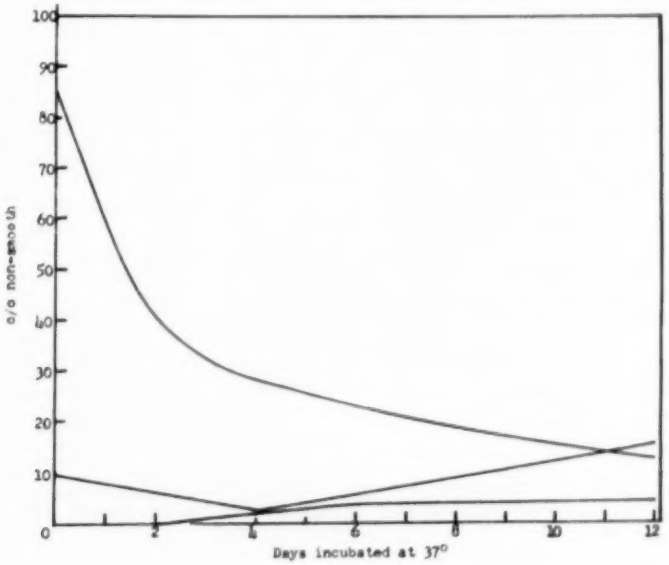

Fig. 2—The percentage of nonsmooth colonial types of Brucella abortus in tryptose-glucose liquid medium in Erlenmeyer flasks.

ble for the suppression of growth of non-S cells has not been determined.

In this discussion, non-S types of cells have been considered as a group and have been compared and contrasted to S and SI types as a group. There is evidence that variants which arise from non-S types vary in susceptibility to the accumulation of substances in the presence of other non-S variants. After each of five pure non-S types of random selection were inoculated in the mediums and incubated for eight days, the cell populations were found to vary from 100 per cent of the original type to 90 per cent new variants of the type inoculated into the medium.

Percentage viability (No. of viable cells/total No. of cells) was calculated for all conditions studied: cultures in test tubes, flasks, conditions of no or slow dissociation, rapid dissociation. The range of viability was wide but could not be correlated with rates of growth or with dissociation. In general, the viability in test tubes was about 45 per cent of the total cell population and in flasks about 55 per cent. Cultures aerated with pure oxygen were lowest in viability. Viability of cells grown in the presence of glucose, fructose, or sucrose in medium in test tubes was not different from that found in the base tryptose medium. The presence of DL-alanine resulted in a low value.

In the course of these studies, a new lot of tryptose agar was employed. It was noted that non-S type colonies on this lot of agar were not readily differentiated by their colonial characteristics and, when stained with crystal violet, the colonies failed to develop their usual characteristic colors. The new lot of tryptose agar contained thiamin hydrochloride which was proved to be responsible for the altered appearance of non-S variants. Single colonies transferred from the thiamin-agar to standard tryptose agar appeared normal and developed characteristic colors when stained with crystal violet. Standard tryptose with thiamin added before autoclaving produced non-S colonies with the same appearances as colonies grown on the new lot of tryptose agar.

SUMMARY

The dissociation of S and SI types of *Brucella abortus* in liquid mediums can be prevented or significantly lowered by an adequate supply of air or oxygen to the culture. This can be accomplished by agitation, by aeration with oxygen, or by growing the cells in a shallow layer of medium. The presence of glucose, fructose, sucrose, or DL-alanine in liquid medium in test tubes increased the dissociation of S types of *Br. abortus*, but in the presence of these agents in a shallow layer of medium in Erlenmeyer flasks, there was little dissociation observed.

The growth conditions which favored stable S and SI colonial types were also suitable for rapid multiplication of those cells and also for non-S colonial types in the absence of S types. In cultures inoculated with mixed cell populations, however, the growth of non-S types of cells was suppressed in a shallow layer of medium.

The results of this study show that the atmosphere and conditions under which cells of *Br. abortus* are grown in liquid mediums are factors as important as metabolites in the causation and prevention of dissociation. Furthermore, it is not possible to evaluate the influence of metabolites on dissociation without considering the atmospheric conditions under which the cells are multiplying.

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Physiological Characteristics Differentiating *Vibrio Fetus* and Other Vibrios

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Vibrio fetus is the etiological agent of a type of infectious abortion in cattle and sheep and of enzootic sterility in cattle. *Vibrio jejuni* and *Vibrio suis* are spirilla encountered in dysenteries of cattle and swine, respectively. *Vibrio metschnikovii* produces an intestinal, cholera-like infection in fowl. A number of unidentified microorganisms, morphologically similar to *V. fetus*, have been isolated from the posterior urogenital tract of cattle and from the intestinal tract of both cattle and swine. Recently, organisms similar to if not identical with *V. fetus* have been cultured from the blood of human patients with endocarditis, placentitis, and from a cheek pustule in man.[16]

Investigative efforts[1, 6, 9–11, 14, 15] have been directed almost exclusively toward elucidation of the transmission, diagnosis, and therapy of animal vibriosis. Some attempts have been made to characterize or systematically classify the vibrios involved. Of the group, *V. fetus* has received the most scrutiny. The studies of Price *et al.*[12] indicate that *V. fetus* does not produce indole, usually reduces nitrate, and fails to grow in nutrient gelatin medium (Baltimore Biological Laboratory). *Vibrio fetus* does not produce demonstrable acid in mediums containing lactose, glucose, sucrose, maltose, xylose, mannitol, inositol, and sorbitol. They also report that some *V. fetus* strains, which grew in litmus milk, reduced the litmus. Three serologically unrelated strains isolated from bovine semen and vaginal mucus produced hydrogen sulfide.[12]

Bryner and Frank[4] stated that there appeared to be two distinct types of vibrios of genital tract origin. Distinction was made on the basis of the catalase and hydrogen sulfide tests:

(1) the catalase positive and hydrogen sulfide negative type which was considered to be characteristic *Vibrio fetus* and

(2) the catalase negative and hydrogen sulfide positive type which was not incriminated as a cause of abortion and infertility in cattle.

Serological studies[4, 12] indicated that there was little or no relationship between the two types. For this reason, the following physiological studies were conducted. There is a definite and continued need for further investigations on the differentiation of pathogenic members of the genus. It is equally important that means be developed to distinguish between the pathogenic and potentially pathogenic forms from the saprophytic vibrios found in soil and water.

Experimental Methods

Sixty-six cultures of vibrios of diverse origins were employed in the investigation (table 1). The stock cultures were maintained in screw-cap culture tubes (125 mm. by 20 mm.) containing ap-

TABLE 1—Vibrio Culture Collection

Organism	Source	No. of Strains
Vibrio fetus	Bovine (vaginal mucus, aborted fetuses and semen)	31
Vibrio fetus	Ovine (aborted fetuses)	9
Vibrio sp.	Bovine (vaginal mucus or semen; not incriminated as causing abortion and infertility)	8
Vibrio fetus	Human (R. Vinzent, Le Havre, France, and E. O. King, C.D.C.,* Chamblee, Ga.)	8
Vibrio sp.	Swine dysentery (K. E. Price, Maryland)	1
Vibrio jejuni	Bovine dysentery outbreak A.T.C.C.** No. 11734, Wyoming State Veterinary Laboratory	1
Vibrio jejuni	Bovine dysentery outbreak (C. C. Cunnick, Kansas City, Mo.)	1
Vibrio comma (Inaba)	University of Wisconsin, Department of Medical Microbiology	1
Vibrio comma (Ogawa)	University of Wisconsin, Department of Medical Microbiology	1
Vibrio metschnikovii	A.T.C.C. No. 7708	1
Vibrio cuneatus	A.T.C.C. No. 6972 (from soil)	1
Vibrio percolans	A.T.C.C. No. 8461, isolated from aqueous hay infusion	1
Vibrio rubicundus	A.T.C.C. No. 8361, isolated from a trickling filter	1
Vibrio tyrogenus	A.T.C.C. No. 7085 from cheese	1

* Communicable Disease Center; ** American Type Culture Collection.

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proximately 15 ml. of Brucella broth (Albimi)® containing 0.1 per cent agar. This medium henceforth will be referred to as semisolid medium. Stock cultures were incubated at room temperature for *Vibrio percolans* and *Vibrio rubicundus* and at 37 C. for the other vibrios. Duration of incubation was 24 to 48 hours.

Cultures 24 to 48 hours old were used as inoculums in all of the following physiological activity examinations. Adequate controls were maintained for the test procedures.

Production of Hydrogen Sulfide.—Approximately 15 ml. of semisolid medium were dispensed in screw-cap tubes. Whatman No. 1 filter-paper strips, which were soaked in a saturated lead acetate solution, dried, and sterilized, were inserted in the tubes to test for evolution of hydrogen sulfide. The cultures were incubated aerobically at 37 C. A darkening of the lead acetate strips after 72 hours was considered evidence of hydrogen sulfide production.

Catalase Test.—After five days of incubation of the semisolid medium cultures, 1 ml. of a 3 per cent hydrogen peroxide (U.S.P.) solution was dispensed in each tube and immediately inverted. If peroxide decomposition occurred, as evidenced by immediate and rapid evolution of bubbles, the strain was considered catalase positive.

Reduction of Nitrates.—Semisolid medium containing 0.1 per cent sodium nitrate was employed to test for nitrate reduction. The cultures were incubated for five days and then α-naphthylamine and sulphanilic acid were added to detect nitrate reduction.[8]

Urease Activity.—The organisms were tested upon Christensen's urea medium slants[5] which contained the additional necessary growth factors per liter:

Nicotinic acid	1.0 mg.
Thiamine HCl	1.0 mg.
Calcium pantothenate	0.2 mg.
Pyridoxal HCl	10.0 mg.
Magnesium citrate	50.0 mg.
$FeCl_2$	0.5 mg.
$MnCl_2$	0.5 mg.
Biotin	5.0 μg.

A standard loopful (ca. 5 mm. dia.) of a 48-hour-old semisolid broth inoculum was used to streak the surface of each urea medium slant. Urea slant cultures, with the exception of known aerobic strains or species, were incubated for one week in an atmosphere containing 225 mm. of mercury of air and 475 mm. of mercury of helium.[10] Another set of urea medium slants was inoculated with 2 loopfuls of the 4-day-old growth of Vibrio from a Brucella agar (Albimi)® slant and incubated under the same conditions as described. The slants were examined daily for seven days for evidence of growth and pH change of the medium.

Action in Litmus Milk.—Fresh, unpasteurized skim milk was used. Litmus solution (20 Gm. of litmus powder dissolved in 100 ml. of distilled water and filtered) was added to give a medium lavender color. Approximately 15 ml. of this medium were dispensed in tubes and sterilized. After inoculation, paraffin was poured over the partially inserted cotton plugs in order to prevent loss of moisture. Incubation was at 37 C. The tubes were examined periodically during one month.

Indole Production.—The test for indole was performed by using the procedure of Goré and Gnezda.[6] The semisolid medium cultures were tested after incubation for 24, 48, 72, 96 hours, and 7 days.

Liquefaction of Gelatin.—Bacto nutrient gelatin (Difco) containing 0.1 per cent agar as well as semisolid medium containing 2, 3, and 4 per cent Bacto gelatin were used to ascertain whether cultures were able to liquefy gelatin. The cultures were placed in a refrigerator to determine whether or not the gelatin would solidify after incubation for periods as long as three months.

Optimum Concentration of Agar for the Cultivation of Vibrio Fetus and Related or Unrelated Vibrios in a Fluid Medium.—Brucella broth (Albimi)® and Brucella broth (Albimi)® containing 0.025 per cent, 0.05 per cent, 0.075 per cent, 0.1 per cent, 0.2 per cent, 0.3 per cent, and 0.4 per cent agar were used to determine the concentration of agar necessary for optimal growth in a semisolid type medium. Final observations for visible growth were made after seven days' incubation at 37 C. under aerobic conditions.

pH Tolerance.—Hydrochloric acid or sodium hydroxide was added to semisolid mediums to increase or decrease the hydrogen ion concentration, respectively. Tolerance determinations, expressed as evidence of visible growth, were made at pH 4.9, 5.4, 6.0, 6.5, 7.5, 8.0, 8.4, 8.8, and 9.3. The cultures were incubated aerobically at 37 C. for two weeks.

Dextrose Tolerance.—Semisolid medium to which was added various percentage amounts of dextrose was employed. The mediums were dispensed in screw-cap tubes and sterilized in the autoclave for 15 minutes at 11 lb. of pressure. Following inoculation, the cultures were incubated aerobically and observed periodically for growth during a period of two weeks.

Sodium Chloride Tolerance.—Semisolid medium containing various percentage amounts of sodium chloride was used to determine the salt tolerance of the different vibrios. The mediums were prepared in 1-liter volumetric flasks and sterilized; the lost moisture was replaced with sterile, distilled water. The mediums were dispensed aseptically in approximately 15-ml. amounts in screw-cap tubes. The cultures were incubated aerobically at 37 C. and observed frequently for growth during a period of two weeks.

RESULTS AND DISCUSSION

Sixty-six Vibrio cultures isolated from lower animals and man as well as from soil, water, and cheese were studied.

Eight cultures produced hydrogen sul-

TABLE 2—Physiological Activities and Growth Requirements of Vibrios of Various Origins

Organism	No. of strains	Catalase production	Growth in semisolid broth containing 3.5 per cent NaCl	Nitrate reduction	Presence of urease	Action in litmus milk	Action in litmus milk containing 0.1 per cent agar (4 days' incubation)	Growth in semisolid broth containing 8 to 11.5 per cent dextrose
Vibrio fetus (bovine)	31	+	—	+	—	No growth	Alkaline zone at surface; reduction of litmus	+
Vibrio fetus (ovine)	9	+	—	+	—	No growth	Alkaline zone at surface; reduction of litmus	+
Vibrio sp. (bovine)*†	8	—	+	+	—	No growth	Alkaline zone at surface; reduction of litmus	+
Vibrio fetus (human)	8	(7+, 1—)	—	+	—	No growth	Alkaline zone at surface; reduction of litmus	+
Vibrio sp. (swine dysentery)	1	+	—	+	—	No growth	Alkaline zone at surface; reduction of litmus	+
Vibrio jejuni A.T.C.C. No. 11734	1	+	—	—	—	No growth	Alkaline zone at surface; reduction of litmus	— (Tolerates maximal 2%)
Vibrio jejuni C. C. Cunnick	1	+	+	—	—	Slightly alkaline	Alkaline zone at surface; reduction of litmus	+
Vibrio comma Inaba and Ogawa**	2	Slight	+	+	Slight	Acid, soft curd, proteolysis, reduction	Alkaline zone at surface; reduction of litmus; slight proteolysis	+ (Tolerates 13%)
*Vibrio metschnikovii***	1	Slight	+	—	—	Acid	Alkaline zone at surface; reduction of litmus	+
Vibrio cuneatus	1	+	+	—	+	Alkaline, 100% proteolysis	Alkaline zone at surface; reduction of litmus	+ (Tolerates 13%)
Vibrio percolans	1	+	—	+	+	Alkaline, slight proteolysis	Alkaline zone at surface; reduction of litmus	+ (Tolerates 13%)
Vibrio rubicundus	1	+	—	—	+	Alkaline	Alkaline zone at surface; slight reduction of litmus	+ (Tolerates 13%)
Vibrio fetogenus	1	+	+	+	+	Alkaline	Alkaline zone at surface; slight reduction of litmus	+ (Tolerates 13%)

* Vaginal mucus and semen organisms not incriminated as causing abortion and infertility; ** indole positive, other vibrios indol negative.
† Produced hydrogen sulfide.

fide (table 2). These had been isolated from the bovine genital tract or bovine semen and had not been incriminated as causing abortion and infertility in cattle. The eight cultures and one human pathogen did not produce catalase. Catalase positive vibrios have been isolated from bovine vaginal mucus and bull semen.[4, 7] The remaining cultures showed vigorous catalase reactions except for *Vibrio comma* and *V. metschnikovii* which possessed only slight activity. Vibrios isolated from soil, water, and cheese were also catalase positive. This finding leads one to question the advisability of identifying isolates from the bovine and ovine posterior urogenital tract as *V. fetus* on the basis of catalase production alone.

The reduction of nitrates may be a physiological activity which might be used to differentiate the vibrios isolated from bovine and ovine aborted fetuses and the reproductive tract, and from the vibrios associated with intestinal disorders of cattle. The former group reduces nitrates, while the latter does not. *Vibrio metschnikovii* as well as the soil and water vibrios *(V. cuneatus* and *V. rubicundus)* do not reduce nitrates to nitrites.

Because of the fastidious growth requirements of *V. fetus,* certain vitamins and minerals [2] were added to Christensen's urea medium [5] to obtain best possible conditions for growth. Definite urease activity was observed within twenty-four hours for *V. cuneatus, V. percolans, V. rubicundus, V. tyrogenus* and lesser activity within seven days for the strains of *V. comma.* The other cultures were urease negative after seven days. Urease activity apparently may be utilized to differentiate these vibrios from the common animal pathogens of the genus.

Except for *V. comma* and *V. metschnikovii* all Vibrio cultures were indole negative. Seven cultures produced reactions in litmus milk (table 2), but *V. fetus* and related Vibrios from the bovine urogenital tract of cattle, as well as the hog dysentery Vibrio, displayed no discernible change. Bergey [3] and Price [12] report no growth of *V. fetus* in nutrient gelatin (Baltimore Biological Laboratory). Growth was not obtained when cultures of *V. fetus* were inoculated in Bacto nutrient gelatin; however, when 0.1 per cent agar was added, this medium supported moderate to good growth of all vibrios examined. Except for *V. metschnikovii, Vibrio cuneatus, Vibrio tyrogenus,* and two strains of *V. comma,* Vibrio cultures did not liquefy gelatin. It was thought that the increased consistency of the fluid medium sustained a growing inoculum at optimal microaerophilic conditions.

Twenty-nine of 48 Vibrio cultures of ovine, bovine, and human origin grew optimally in semisolid medium containing 0.075 per cent agar. Fifteen cultures had comparable growth in semisolid medium with either 0.075 per cent or 0.1 per cent agar, while four cultures grew best when the agar concentration was 0.1 per cent. It appears that agar concentrations of 0.1 per cent or 0.075 per cent are optimal for this particular medium since the conditions for luxuriant growth are provided.

Since it was found that an optimum amount of agar was required to maintain Vibrios in these mediums, 0.1 per cent agar was added to litmus milk. Growth was obtained for 48 *V. fetus* cultures, eight related Vibrio cultures isolated from the bovine unrogenital tract of cattle (catalase negative, hydrogen sulfide positive) and one Vibrio culture isolated from a hog. All these cultures produced a distinct alkaline zone of 1 to 6 mm. in depth just below the meniscus (in screw-cap tubes 125 mm. by 20 mm.) after 48 hours. This development was followed by a complete reduction of the remaining, previously unchanged litmus after 96 hours of incubation at 37 C. Again it appeared that the addition of agar produced optimum environmental conditions for growth of vibrios in a fluid medium.

The optimal temperature for growth was 37 C. for all Vibrios except *V. percolans* and *V. rubicundus* which had a range of 28 C. to 30 C. The highest temperatures which would permit growth of most Vibrios were 40 C. to 43 C. However, one strain of *V. jejuni* (C. C. Cunnick) grew at 45 C. while *V. percolans, V. jejuni* (A.T.C.C.), *V. cuneatus,* and *V. rubicundus* did not grow at 40 C.

Animal pathogens of the catalase positive, hydrogen sulfide negative group could be cultivated, as indicated by visible growth, at 15 C. The catalase negative, hydrogen sulfide positive group isolated from bovine vaginal mucus and bovine semen did not grow at 15 C. but did grow at 20 C. Visible

growth was observed for *V. metschnikovii, V. cuneatus, V. percolans,* and *V. rubicundus,* at 5 C. On the basis of growth at 5 C., 15 C., and 20 C. differentiation of species or types could be made.

Vibrio cuneatus, V. percolans and 24 of the Vibrio cultures isolated from cattle, sheep, swine, and man produced visible growth in semisolid medium at pH 5.4 at 37 C. for two weeks. All the Vibrio cultures grew at pH 6.0 except for *V. jejuni* (A.T.C.C.) which could be cultivated at pH 6.5 but not at pH 6.0. *Vibrio comma, V. metschnikovii, V. cuneatus, V. percolans,* and *V. tyrogenus* grew at pH 9.3, whereas the two strains of *V. jejuni,* five cultures of *V. fetus* (ovine origin) and five cultures of the catalase negative, hydrogen sulfide positive vibrios grew at 8.8. All the vibrio cultures grew at pH 8.4.

Vibrio cultures in semisolid medium, initial pH 6.9, after two weeks of incubation at 37 C. produced a terminal pH range of 7.2 to 8.6. No differentiation among species or types was evident.

Differentiation among the Vibrio cultures isolated from cattle and sheep on the basis of their ability to grow in a semisolid medium containing different amounts of dextrose (table 2) was not possible except for *V. jejuni* A.T.C.C. No. 11734 which grew at no higher than the 2 per cent sugar concentration. *Vibrio comma* and *V. cuneatus* had abundant growth in semisolid medium containing 13 per cent dextrose. The data show no apparent differentiation of vibrios isolated from the reproductive tract of cattle or sheep based on dextrose tolerance.

Vibrio fetus strains of bovine, ovine, and human origin (catalase positive, hydrogen sulfide negative) could be cultivated in the presence of a maximal concentration of 2.5 per cent sodium chloride. The hog dysentery strain, *V. percolans, V. rubicundus,* and one strain of *V. jejuni* behaved similarly. Vibrios isolated from bovine vaginal mucus and semen (catalase negative, hydrogen sulfide positive) were cultivated with ease in mediums with sodium chloride concentrations of 3.5 per cent and higher. Single strains of *V. jejuni, V. comma, V. metschnikovii, V. cuneatus,* and *V. tyrogenus* also grew in 3.5 per cent sodium chloride semisolid medium. Sodium chloride tolerance during growth offers an additional means of differentiating Vibrio species and types.

The data on the physiological and growth characteristics of vibrios of diverse origins are summarized in table 2.

SUMMARY

Certain physiological activities and growth characteristics of 66 cultures of Vibrio, both pathogenic and nonpathogenic, of diverse origin, were studied.

1) All cultures were catalase positive except for the eight Vibrio strains which were isolated from bovine vaginal mucus and semen and one strain of *V. fetus* of human origin.

2) The 56 strains of *V. fetus* of diverse origin, one strain of Vibrio isolated from a hog, and two strains of *V. comma* were nitrite positive. Two strains of *V. jejuni, V. metschnikovii, V. cuneatus,* and *V. rubicundus* did not reduce nitrates to nitrites.

3) All Vibrio cultures were indole negative except for *V. metschnikovii* and two strains of *V. comma.*

4) In litmus milk containing 0.1 per cent agar, all *V. fetus* and related organisms showed growth within 48 hours. All 66 cultures produced a distinct alkaline zone at the surface. While the surface alkaline zone persisted, complete reduction of the remaining litmus occurred after 96 hours.

5) *Vibrio cuneatus, V. percolans, V rubicundus, V. tyrogenus,* and *V. comma* possessed the enzyme, urease; the remaining cultures did not.

6) Growth without liquefaction of a modified nutrient gelatin medium was obtained for all *V. fetus* and related cultures.

7) Agar concentrations of 0.1 per cent or 0.075 per cent in a semisolid medium are optimal for *V. fetus* and related organisms.

8) Separation of types of species of Vibrio could be made on the basis of their ability to grow at 5 C., 15 C., and 20 C. The maximum temperatures for growth of strains of *V. fetus* and related organisms were 40 C. to 43 C. The minimum temperature for growth of *V. fetus* was 15 C. Vibrios isolated from bovine vaginal mucus or bovine semen (catalase negative, hydrogen sulfide positive) were not cultivatable at 15 C., whereas growth occurred at 20 C.

9) All Vibrio cultures with the exception of *V. jejuni* (A.T.C.C.) showed best visible growth in semisolid medium adjusted to pH 6.0 to pH 8.4.

10) Vibrios isolated from the reproductive tract of cattle and sheep could not be differentiated by their tolerance of added dextrose in semisolid medium.

11) Sodium chloride tolerance during growth offers an additional means of separating various strains of *V. fetus* (catalase positive, hydrogen sulfide negative) from those of Vibrio sp. (catalase negative, hydrogen sulfide positive) of bovine origin. *Vibrio fetus* strains did not withstand 3.5 per cent sodium chloride concentration in mediums while the related Vibrio sp. grew well.

Vibrio fetus can be defined as catalase positive, hydrogen sulfide negative, and unable to grow on initial isolation in a medium containing 3.5 per cent sodium chloride.

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